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Contaminant Concentrations and Biomarker Activity in Wild Mussels near Point Sources of Contaminants in the Lower Bay of Fundy

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Contaminant Concentrations and Biomarker Activity in Wild Mussels near Point Sources of Contaminants in the Lower Bay of Fundy

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ABSTRACT

An investigation of the potential for endocrine disruption, the incidence of leukemia, and contaminant levels in bivalve molluscs near potential sources of contaminants in southwest Nova Scotia (NS) and New Brunswick (NB) was undertaken in November and December 2002. Wild blue mussels (*Mytilus edulis*) and sediments were collected near finfish aquaculture areas, municipal waste water sites, industrial sites (ferry terminal, pulp mill, fish processing plant), as well as areas for which large point source discharges could not be identified. Collection sites were located in the lower Bay of Fundy, along the Annapolis Basin in NS, as well as in Passamaquoddy Bay, NB and its surrounding area.

Mussel condition indices were different between sites, with some of the highest condition indices found at aquaculture sites ($p < 0.001$). Metals in soft tissues were not elevated above those found at less impacted areas and among organics only (PCB's, chlordane, DDT, DDE and DDD) were found to be slightly elevated at the ferry station, pulp mill and fish plant sites.

While leukemia rates were indistinguishable from background rates in NS, there were elevated levels of leukemic cells near the areas of anthropogenic activity in NB. These areas of anthropogenic activity included the aquaculture sites, sewage treatment plant, ferry station, pulp mill and fish plant. Estrogenicity in sediments was measured by estradiol activity using the YES assay was low (generally less than 15% and often less than 5%) for both less impacted and treatment sites in water-extracted sediments. Analysis of vitellogenin-like proteins (vitellins) in soft mussel tissue revealed significant differences in vitellin levels between sites. Also, values were lower at treatment sites than the less impacted sites with the exception of the sewage treatment site. The sewage treatment site showed increased levels of vitellogenin-like proteins and gametogenic activity, as determined by aspartate transcarbamoylase activity. The discovery of significant differences between sites warrants further investigation to verify changes to the health status of mussel populations.

RÉSUMÉ

Nous avons effectué une étude de la perturbation des fonctions endocriniennes, de l'incidence de la leucémie et des teneurs en contaminants chez des mollusques bivalves trouvés près de sources potentielles de contaminants dans le sud-ouest de la Nouvelle-Écosse (N.-É.) et au Nouveau-Brunswick (N.-B.) en novembre et décembre 2002. À cette fin, nous avons prélevé des individus sauvages de moule bleue (*Mytilus edulis*) et des sédiments à proximité de piscicultures, de stations municipales d'épuration des eaux d'égout et d'emplacements industriels (gare maritime, usine de pâtes, usine de transformation du poisson), ainsi qu'à des endroits où les sources ponctuelles de polluants n'étaient pas identifiables. Les points de prélèvement étaient situés à l'embouchure de la baie de Fundy et le long du bassin d'Annapolis, en N.-É., ainsi que dans la baie Passamaquoddy et ses environs, au N.-B.

Les indices de condition des moules variaient selon les sites; un certain nombre des indices les plus élevés ont été trouvés à l'emplacement des installations piscicoles échantillonnées ($p < 0,001$). Les teneurs en métaux dans les tissus mous des moules n'étaient pas plus élevées que les teneurs établies pour les endroits moins perturbés, et dans le cas des composés organiques, seuls le BPC, le chlordane, le DDT, le DDE et le DDD montraient des teneurs légèrement élevées à l'emplacement de la gare maritime, de l'usine de pâtes et de l'usine de transformation du poisson.

Bien que le taux d'incidence de la leucémie chez la moule bleue était indifférenciable du taux naturel en N.-É., nous avons décelé des concentrations élevées de cellules leucémiques chez les moules prélevées aux endroits situés à proximité de zones d'activité anthropique au N.-B., y compris les sites aquacoles, la station d'épuration des eaux d'égout, la gare maritime, l'usine de pâtes et l'usine de transformation du poisson. L'oestrogénicité des sédiments, mesurée par l'activité de l'oestradiol d'après l'essai YES, était faible (généralement moins de 15 % et souvent moins de 5 %) dans les sédiments prélevés dans l'eau, tant aux sites moins perturbés qu'aux sites de traitement des eaux usées. La mesure des protéines similaires à la vitellogénine (vitellines) présentes dans les tissus mous de moule a révélé des différences significatives dans les concentrations de vitelline entre les sites. En outre, ces concentrations étaient moins élevées aux sites de traitement des eaux usées qu'aux sites moins perturbés, à l'exception de la station d'épuration des eaux d'égout. À cet endroit, les concentrations en protéines similaires à la vitellogénine étaient élevées, tout comme l'activité gamétogénique, établie d'après l'activité de l'aspartate transcarbamoylase. La découverte de différences significatives entre les sites appelle d'autres études pour établir si l'état de santé des populations de moules a décliné.

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1.0 INTRODUCTION

Coastal zones are typically areas of industrial and municipal activity and are therefore subject to various inputs of contamination from anthropogenic sources. The risks associated with chemical inputs into estuarine and coastal environments are numerous. One emerging area of concern is the impact of endocrine disrupting chemicals (EDC's) on human and wildlife health. Endocrine disrupters are exogenous substances or mixtures that alter the function(s) of the endocrine system by interfering with the production, release, transport, metabolism, binding, action or elimination of natural hormones, consequently causing adverse health effects in an intact organism or its progeny. These chemicals interfere with hormonal homeostasis in organisms and the regulation of developmental processes (Kavlock et al, 1996). Possible adverse human health effects include breast cancer and endometriosis in women, testicular and prostate cancers in men, abnormal sexual development, reduced male fertility, alterations in pituitary and thyroid gland functions, and neurobehavioural effects (U.S. EPA, 1997). In addition to human health, wildlife health may also be affected. If left unchecked, EDC's may have population level effects (Servos et al, 2001). Documented chemicals of concern include organo-chlorine compounds, such as 1, 1, 1-trichloro-2, 2-bis(*p*-chlorophenyl)ethane (DDT), DDT metabolites, polychlorinated biphenyls (PCB's), dioxins, and naturally occurring plant estrogens (Kavlock et al, 1996). Some of the best known examples of adverse effects on wildlife include DDT-associated thinning of bird egg shells, organotin (antifoulant) induced imposex in marine snails inhabiting areas of heavy shipping traffic, impaired reproduction and development of fish exposed to pulp and paper mill effluent, and feminization of fish near municipal effluent outlets (DeGuise et al, 2001).

Finfish aquaculture is an expanding industry in the Atlantic Region and is known to use a variety of chemicals (Muise et al, 2000). However, the risk of environmental effects from their use is still unknown. Other industrial sites which discharge to the marine environment, such as municipal wastewater effluent and pulp mill effluents have the potential to cause adverse effects. In order to investigate the potential impact of anthropogenic chemical releases, a study involving wild blue mussels was undertaken by Environment Canada in cooperation with Atlantic Coastal Action Plan (ACAP) groups, St. Croix Estuary Project (SCEP) and the Clean Annapolis River Project (CARP).

The wild blue mussel (*Mytilus edulis*) is the indicator organism in this study. The advantages of its use include that fact that wild blue mussels are indigenous to, and abundant in, the region of the study and there is a large body of scientific literature on *Mytilus* species. They are easy to collect and process, they are commercially important, and they are sessile filter-feeders that are chronically exposed to numerous environmental pollutants such as organochlorines and heavy metals, which they then concentrate (Jones et al, 1998).

Biomarkers of reproduction, such as vitellin (egg yolk protein) levels have the potential to indicate endocrine disruption. Vitellogenin is the precursor to vitellin. Vitellogenesis is the process whereby egg proteins are produced. It entails both the synthesis of vitellogenin (in the liver for vertebrates and in the gonad for some invertebrates) and its uptake into growing oocytes where it is stored as yolk. Subsequently, vitellin serves as the food reserve for the developing embryos. The expression of the vitellogenin gene, and hence the synthesis of vitellogenin, is under multihormonal control, with 17β–estradiol playing the dominant role (Li et al, 1998). As 17β–estradiol concentrations rise, vitellogenin concentrations rise to the level at which an ovary containing thousands of oocytes is stimulated to grow. In contrast, vitellogenin levels in males are low, presumably because circulating estrogen levels are too low to trigger expression of the vitellogenin gene. Vitellogenin production can be induced in males if exposed to natural and synthetic estrogens (Purdom et al, 1994). The huge range in potential vitellogenin concentrations serves as the basis for the biomarker assay. Significantly increased levels of vitellins in female mussels, and especially in male mussels, are indicative of the presence of environmental estrogens and of endocrine disruption taking place. The yeast estrogen screen (YES) assay detects the presence of estrogenic substances in environmental samples.

Leukemia has been found to be more prevalent in clams inhabiting polluted waters compared those from pristine areas, suggesting an environmental component to this disease (Barker, et al, 1997). This study attempted to measure contaminant levels, endocrine disrupting potential and mussel leukemia in environmental samples collected near a variety of anthropogenic contaminant sources.

2.0 METHODS

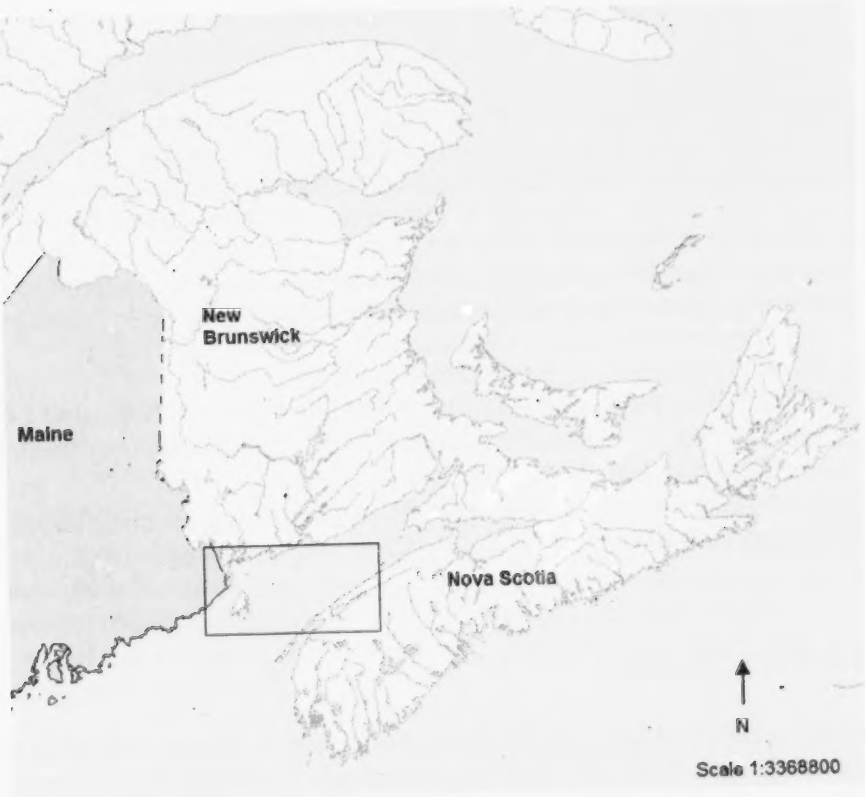
2.1 Location of Field Sites

Field sites were selected based on the type of anthropogenic activity, and thus the effluent type present, the presence of mussels at each of the required sampling distances and the accessibility of shoreline. All mussels were collected as close to the time of low tide and as close to the water's edge as possible. Wild blue mussels and sediments were collected from aquaculture areas, municipal waste water sites, near industrial sites (pulp mill, fish processing plant), and less impacted areas. Field sites were located along the Annapolis Basin in NS, and in and adjacent to Passamaquoddy Bay in NB, in the respective jurisdictions of the two ACAP groups.

The field sites in NS included: (1) a site in Karsdale (near Port Royal), which had no identified sources of anthropogenic chemical inputs other than sparse residential buildings and was thus considered to be less impacted site; (2) a

secondary sewage treatment plant site in the town of Digby (summer population 3500 - 4000) and (3) a finfish aquaculture site near Rattling Beach in Digby Gut (18 cages and 15,000 fish). The latitude and longitude of each site is contained in Appendix 1.

Figure 1 indicates the field sites located in NB and NS. Field sites included: (1) a less impacted site in Dead Man's Harbour, which was devoid of industrial activity but had sparse residential buildings; (2) a secondary sewage treatment plant in St. Andrews (summer population 5-6000, winter population 1800); (3) the Lime Kiln finfish aquaculture site in Back Bay; (4) the Kilmarnoc Head finfish aquaculture site in Chamcook (5 fish farms, with approximately 800,000 caged fish in total); (5) a ferry terminal site in Black's Harbour (total number of passengers, both non-commercial and commercial, in 1999: 171,000); (6) a fish plant also in Black's Harbour (with a daily output of 250 metric tonnes of fish in peak season (ie: July, August and September) and 181 metric tonnes in non-peak season) and (7) a pulp mill site in St.George (with an average daily effluent of 489 kg). The latitude and longitude of each site is contained in Appendix 2.



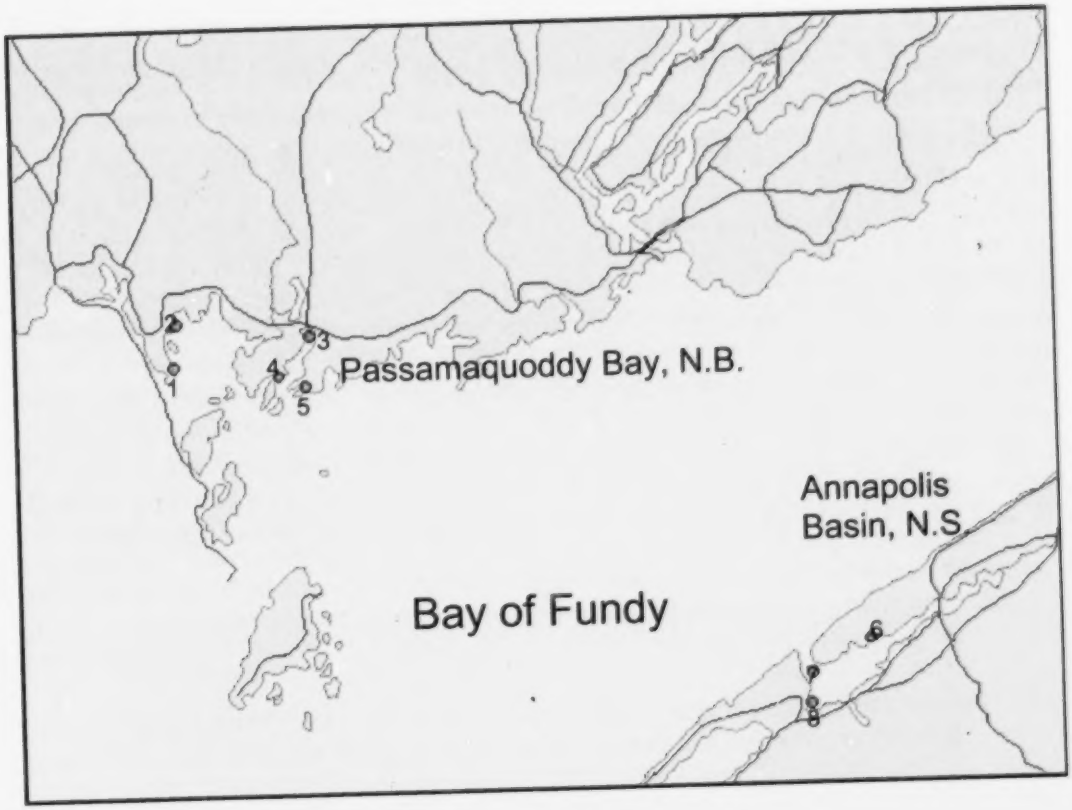


Figure 1: Location of field sites in NS and NB.
[1]Sewage Treatment Plant, St. Andrews, N.B.
[2]Kilmarnoc Head Aquaculture Site, Chamcook, N.B.
[3]Lake Utopia Pulp Mill, St. George, N.B.
[4]Lime Kiln Aquaculture Site, Back Bay, N.B.
[5]Grand Manan Ferry and Fish Plant, Black's Harbour, N.B.; and Less
[6]Impacted Site, Dead Man's Harbour, N.B.
[7]Less Impacted Site, Karsdale, N.S.
[8]Aquaculture Site, Rattling Beach, N.S.
[9]Sewage Treatment Plant, Digby, N.S.

2.2 Collection of Mussels

A minimum of 100 mussels (5-6 cm shell length) were collected at each of the three sampling sites for each effluent type tested. At each effluent output, mussels were collected at the point source (0m) and at 500m and 1000m from the identifiable point of discharge. For both the fish plant and pulp mill sites, only 0m samples were collected. For the aquaculture sites, 0m was situated as close as possible to the cages. Measurements of length, width and height (mm) were

taken on all mussels with Vernier callipers to characterize the size variation in the sample population.

Sixty mussels from each sampling site were randomly chosen and packaged separately for each of the following assays: metal analysis, organic analysis, reproductive biomarkers (ie: extractable gonadal proteins and vitellogenin-like proteins in female mussels), gametogenesis activity (aspartate transcarbamoylase activity) and coprostanol and cholesterol levels. In addition, 30 mussels were separated at the site, kept submerged in ice cooled water taken from each site, transported to the laboratory facilities and immediately assessed for any incidence of leukemia. All mussels were cleaned of external sediment and external growth (ie: algae, other organisms, and byssal threads).

Cleaned mussels destined for reproductive biomarker analysis were placed in large ziplock bags (20/bag; one bag per station), frozen for 24 hours at -20 °C, and shipped to the Environment Canada laboratory at the Centre Saint Laurent in Montreal, Quebec where they were stored at -85 °C until analysed.

Mussels destined for metal analysis were shucked with an acetate wedge. A composite of the soft tissue from each station was placed in a large ziplock bag (20/bag, one bag per station). Another 20 mussels destined for organic analysis were shucked with a stainless steel knife. A composite of the soft tissue from each station was placed in a 1-litre glass mason jar. Samples for both metal and organic analyses were frozen for 24 hours at -20 °C and shipped to the Environment Canada analytical laboratory in Moncton.

At each of the sampling sites for all effluent types tested, sediments were collected with Teflon core tubes and stored frozen until the time of the YES assay.

2.3 Contaminant Analyses

The methods used for analysis of metal and organic contaminants in mussel tissue were those of the Gulf Watch (Jones et al, 1998). Concentrations for metals are reported as dry weight (ug/g) and organic concentrations are reported as wet weight (ng/g). Metal contaminants analyzed included arsenic (As), manganese (Mn), selenium (Se), nickel (Ni), zinc (Zn), lead (Pb), cadmium (Cd), copper (Cu), and cobalt (Co).

Organic contaminants analyzed included PCB (congeners 8, 18, 28, 29, 44, 50, 52, 66, 77, 87, 101, 105, 118, 126, 128, 138, 153, 169, 170, 180, 187, 195, 206, 209), aldrin, chlordane, DDD, DDE, DDT, dieldrin, endosulfan, endrin, heptachlor, benzenehexachloride, methoxychlor, mirex, HCB, Nonachlor, and the sea-lice control agent emmamectin for the aquaculture sites only.

2.4 Leukemia Assessment

The mussels assessed for leukemia were also assessed for condition factor (CF). CF was determined by calculating the ratio between whole organism weight and volume, calculated from length, width and height measurements. Mussels were dissected and the soft tissue dried at 60°C until weights were constant (approximately 48 hours). Wet weight/dry weight ratios were calculated to determine variation in moisture content.

The technique used to assess leukemia described in St-Jean et al. (2003) was followed. Briefly, leukemic haemolymph cells from mussels may be distinguished from haemocytes because they are abnormally large, have a high nuclear-to-cytoplasmic ratio, and are often undergoing mitosis. They are non-adhesive, are non-phagocytic and do not extend pseudopodia (Beckmann et al. 1992; Morrison et al. 1993; Stephens et al. 2001). An important third category of cells, called transitional or intermediate (White et al. 1993; Stephens et al. 2001), are enlarged and have a large nucleus, but extend pseudopodia. These transitional cells progress from normal to fully differentiated leukemia cells (White et al. 1993).

A first bleeding of 0.1 ml was performed using a sterile 23 gauge needle inserted directly in the posterior adductor muscle sinus. The haemolymph was drawn into a dry 3 ml syringe from each animal, and a preliminary live diagnosis was made using phase contrast microscopy by two different readers. When disagreement could not be resolved between the two readers (<10% of case) a third reader would asses the sample. The haemolymph was assessed for: end-stage leukemic cells, transitional cells and normal cells. This assessment was qualitative and ranked leukemia severity according to the following scale: normal (all cells are normal), light (less than 10 % of the cells are abnormal), medium (between 10 and 50 % of the cells are abnormal) and heavy (more than 50 % of the cells are abnormal). Abnormal cellular counts and cases of high haemocyte counts were noted.

To confirm the diagnosis of leukemia, or when the diagnostic was unclear, a second bleeding of 1-1.5 ml was performed and evaluated with monoclonal antibody analysis (MAB). The haemolymph used for MAB confirmation was fixed according to St-Jean et al. 2002 and 2003. The resulting haemolymph/fixative solution was stored in an Eppendorf tube (2ml), numerically coded and sent to Environment Canada where the assays were performed blind.

2.5 Immunofluorescence

Immunofluorescence followed the technique described in St-Jean et al. (2003). Briefly, cells were incubated overnight with monoclonal antibodies 2A4 or 1E10 or normal IgG, after which a 1:100 dilution of rhodamine coupled with rabbit anti-mouse IgG (Molecular Probes) was added to each sample. These samples were evaluated with an Olympus microscope (rhodamine filter). In each analysis, a normal (IgG) control and saline solution were used to control for non-specific binding of the secondary antibody or for a background signal.

The 1E10 antibody binds to a high molecular-weight antigen uniquely present on the cell surface of end-stage leukemia cells (Miosky et al. 1989). The antibody-antigen complex thus formed fluoresces and allows for immediate and unambiguous identification of leukemic cells. The number of fluorescing cells within 200 cells was tabulated and converted to a percentage of total cells (referred tc as leukemia intensity).

2.6 Reproductive Biomarkers

The methods for the chemical analysis of biomarkers of reproduction have been previously described for coprostanol and cholesterol (Gagné et al. 2001), for aspartate transcarbamoylase activity (Mathieu, 1987; Herries, 1967), for alkali labile phosphate (Blaise et al. 2003) and for extractable proteins (Eckelbarger and Davis, 1996).

2.7 Yeast Estrogen Screening

The YES assay for measuring estrogenicity in sediment was conducted according to Burnison (et al. 2003). Sediment samples were thawed at 4°C for 16 hours, then weighed into capped polypropylene microcentrifuge tubes (1.5 ml microtubes, #SPE150-N, Diamed Lab Supplies, Inc. Mississauga, ON). Approximately one gram of sediment was placed into each of two tubes per sample. Reconstituted seawater was added to one tube and methanol to the other in 1 ml aliquots. Extraction was allowed to proceed at 4°C for a minimum of two hours, then the tubes were centrifuged for four minutes at 14,000 rpm (16,200 g) in a microcentrifuge (Eppendorf Centrifuge 5414C, Rotor F45-18-11, Brinkmann Instruments, Inc., Westbury, NY). The supernatant was decanted off and tested by the YES bioassay. A portion of each sediment sample (termed DWA, dry weight aliquot) was weighed, air-dried at 45°C and % water was calculated. The sediment remaining in the tubes (which had been either water or methanol extracted) was also dried and % water calculated. Initially the dry weight of the extracted sediments was calculated using the % water value

determined by the DWA. However, with subsequent extractions the dry weight was determined directly.

The seawater (Instant Ocean Synthetic Sea salt, Aquarium Systems, Mentor, OH) was reconstituted with distilled water. The solution was made using a hydrometer (specific gravity = 1.245, approximately equal to 33 g/L).

Several of the methanol extract samples were toxic to the yeast used in the YES assay. These sediments were re-thawed and 4 to 5 grams of sediment weighed (wet weight). Subsequently, 5 ml of methanol was added and allowed to extract in the fridge. Each sample, after a minimum of two hours extraction time was filtered through Whatman GF/F filters. Filters were pre-weighed so dry weights could be calculated. Two ml of each extract were diluted to a 10% methanol concentration with Milli-Q water and applied to conditioned SPE's (Supelclean LC-18, 6 ml, 0.5 gm, Supelco, Bellefonte, PA). The SPE's were eluted with 70% methanol followed by 100% methanol. Total volume of each extract tested with the YES assay was 2 ml.

The data presented show the percent of 1nM estradiol activity in the sediments as indicated by the activity of beta-galactosidase. If the substance being analyzed was a pure chemical, one could use a standard curve to calculate a true estradiol equivalency. However, in a medium such as the sediment used in the current study, there are various chemicals present, some of which may be estrogenic, some which may be anti-estrogenic, some which may have no estrogenic properties, and others that may be toxic to the yeast and inhibit growth, etc. There are several possible contributing factors to the outcome of the estradiol activity values. Thus, it is impossible to use the data as a quantitative description, it is only qualitative.

2.8 Statistical Methods

Statistical analysis was completed with the software package Systat 10 (2000). Continuous variables were subjected to a one-way analysis of variance to examine the effect of treatment. A Tukey Post-Hoc comparison was used to determine significant differences between sites. P-values below 0.05 were considered to be significant. The Kruskal-Wallis test was used to analysis ranked data.

3.0 RESULTS

3.1 Organic Contaminants in Mussel Tissues

Values are expressed as ng/g on a wet tissue weight basis. At the aquaculture sites, emmamectin benzoate, a parasite control chemical, used in the industry was also analyzed. This chemical was not found at concentrations above the

method detection limit (0.400 ug/g).Other analyzed compounds not above the method detection limit were: PCB congeners 2,4'-dichlorbiphenyl (PCB 8), 2,2',5-trichlorbiphenyl, 2,4,4'-trichlorbiphenyl, 2,4,5-trichiorbiphenyl, 2,2',4,6-tetrachlorbiphenyl, 2,2',5,5'-tetrachlorbiphenyl, 2,2',3,3',4,4',5-heptachlorobiphenyl, 2,2',3,3',4,4',5,5',6-nonochlorobiphenyl, 3,3',4,4',5,5'-hexachlorobiphenyl, and 3,3',4,4',5-pentachlorobiphenyl; aldrin; alpha-benzenehexachloride; alpha-and beta-endosulfan; endrin; heptachlor epoxide; p,p'-methoxychlor; mirex; hexachlorobenzene; and heptachlor. When substances fell below the detection limit, the detection limit was noted on the graph by a dashed line. Measurements below the detection limit were recorded in the graphs as one-half of the detection limit.

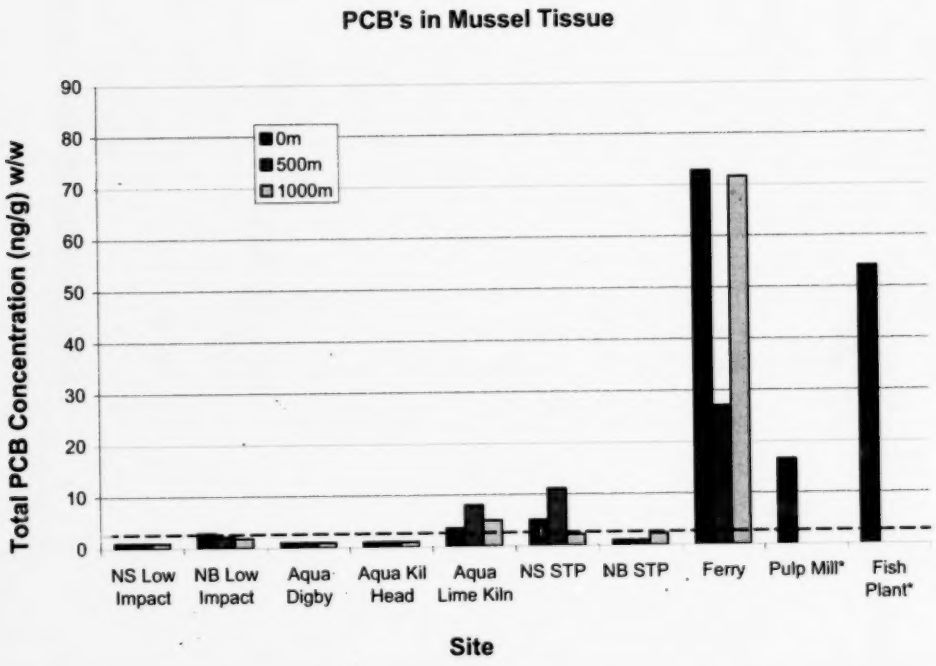


Figure 2: Concentration of total PCB's (ng/g wet weight) in the homogenized soft tissue taken from 20 mussels from each site. The dashed line represents the highest individual congener detection limit of 2.0 ng/g. Measurements below the detection limits have been given a value of 1.0 ng/g.

PCB congeners which showed concentrations above the detection limit were as follows: 2,2',3,5'-tetrachloro-biphenyl; 2,3',4,4'-tetrachlorbiphenyl; 3,3',4,4'-tetrachloro-biphenyl; 2,2',3,4,5'-pentachlorbiphenyl; 2,2',4,5,5'-pentachlorbiphenyl; 2,3,3',4,4'-pentachlorbiphenyl; 2,3',4,4',5-pentachlorobiphenyl; 2,2',3,3',4,4'-hexachlorobiphenyl; 2,2',3,4,4',5'-hexachlorobiphenyl; 2,2',4,4',5,5'-hexachlorobiphenyl; 2,2',3,4,4',5,5'-heptachlorobiphenyl; and 2,2',3,4',5,5',6-heptachlorobiphenyl. PCB's were not above the method detection limit at many of the sites including the less impacted

sites in both NS and NB, and the NS aquaculture site (Digby). Values were as high as 70 ng/g at the ferry terminal site. At the Lime Kiln aquaculture site and sewage treatment plants, values were approximately 10 ng/g or less. Values at the pulp mill and fish plant were 15 ng/g and 45 ng/g respectively. There was no consistent relationship between PCB concentration and distance from any of the sites.

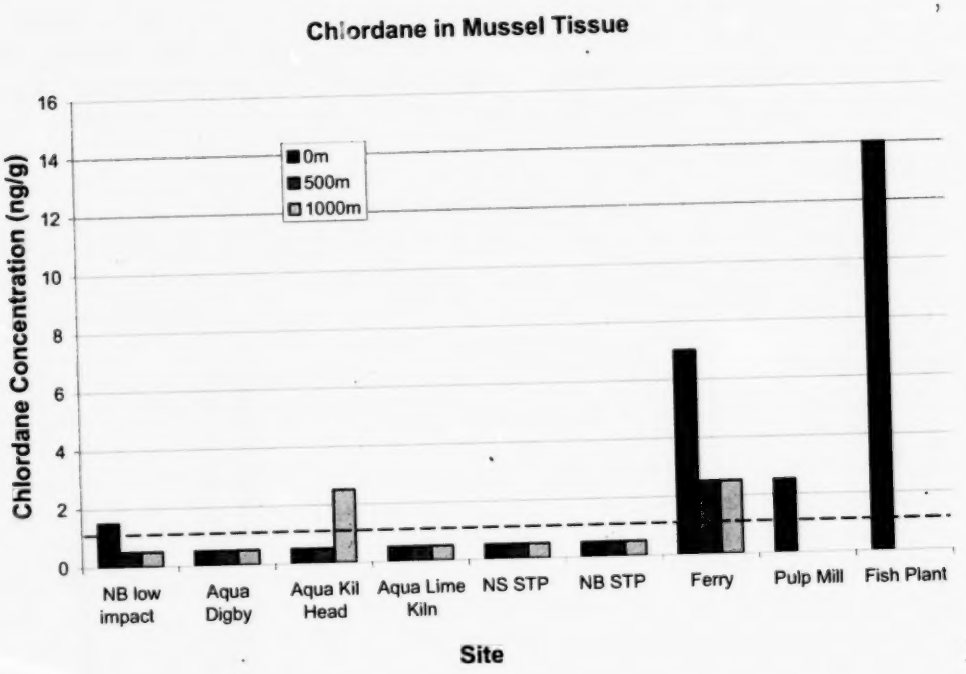


Figure 3: Chlordane concentration (ng/g wet weight) in the homogenized soft tissue taken from 20 mussels from each site.

Chlordane was found in many of the same sites as the PCB's, whereas benzene hexachloride was found at fewer sites (Figure 3). Surprisingly, benzene hexachloride was elevated at the less impacted site in NB.

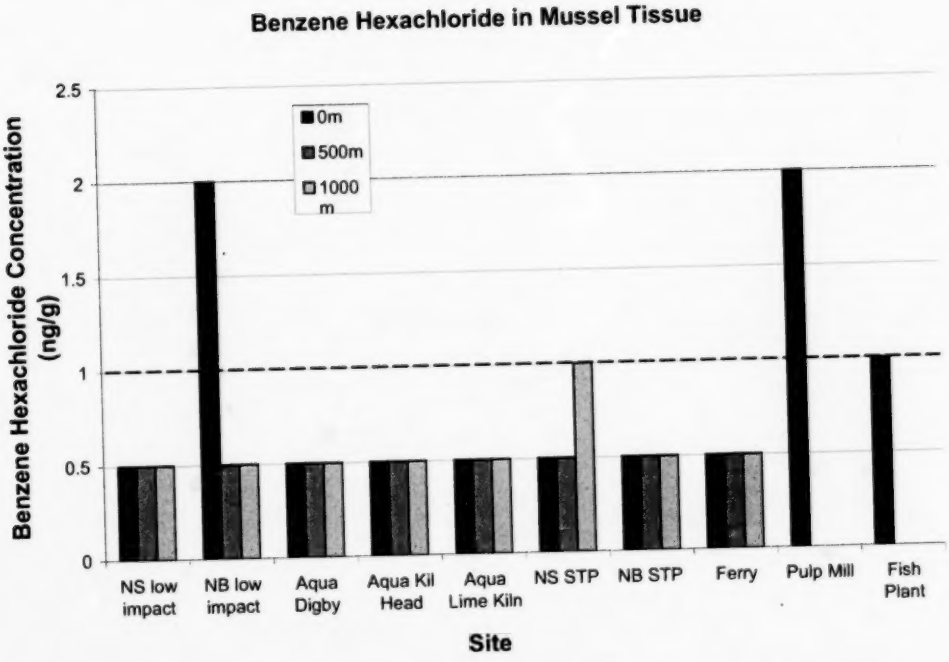


Figure 4: Benzene hexachloride concentration (ng/g wet weight) in the homogenized soft tissue taken from 20 mussels from each site.

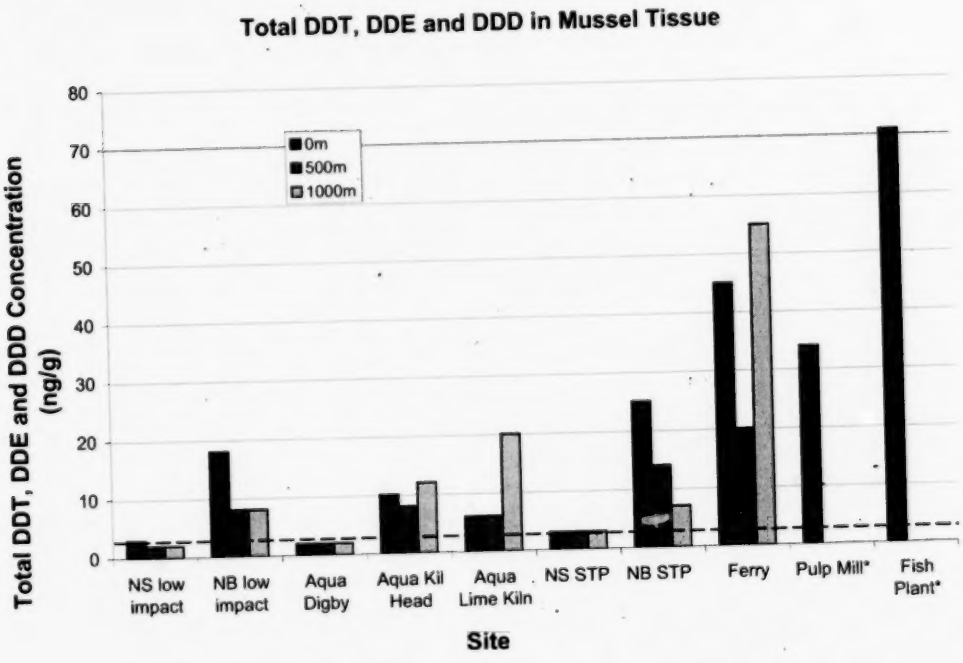


Figure 5: DDT, DDE, and DDD concentrations (ng/g wet weight) in the homogenized soft tissue taken from 20 mussels from each site.
DDT, its metabolites (DDE and DDD), and dieldrin (next graph) were ubiquitous throughout the sample sites (Figure 5). Concentrations of the DDT family of compounds were generally low, often less than 5 ng/g. At the NB sewage treatment plant, the Lime Kiln aquaculture site and the NS and NB less impacted sites, the highest values were between 12 and 22 ng/g. Values for the pulp mill, ferry station and fish plant were as high as 30, 48, and 60 ng/g respectively.

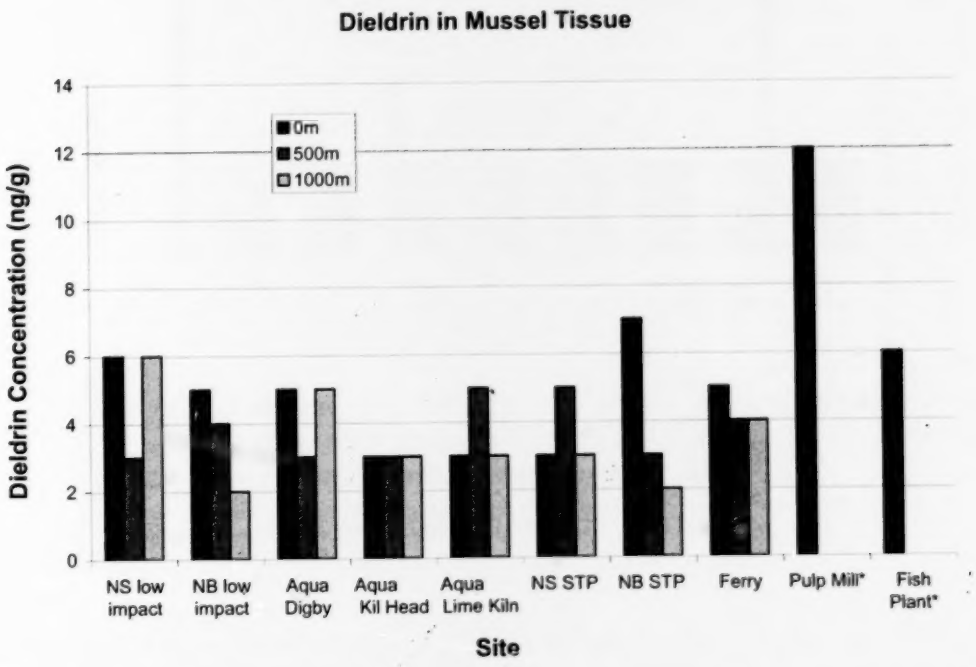


Figure 6: Dieldrin concentration (ng/g wet weight) in the homogenized soft tissue taken from 20 mussels from each site.

Values of Dieldrin were 7 ng/g or less, except at the pulp mill (12 ng/g) (Figure 6).

3.2 Metal Contaminants in Mussel Tissues

Metal contaminants analyzed included arsenic (As), manganese (Mn), selenium (Se), nickel (Ni), zinc (Zn), lead (Pb), cadmium (Cd), copper (Cu), and cobalt (Co). They were expressed on a dry weight basis (ug/g). The metals were ubiquitous (ie: found at all sites and at all distances) and values were above the method detection limit in all cases (Table 1).

Table 1. Metal concentrations (ug/g dry weight) in the homogenized soft tissue taken from 20 mussels from each zero distance site (min and max values for each metal are bolded).

Station	Metal Contaminants								
(Zero Distances Only)	As	Mn	Se	Ni	Zn	Pb	Cd	Cu	Co
NS less impacted	7.5	19.0	2.8	1.5	47.0	2.5	1.0	4.0	**0.8**
NB less impacted	8.3	6.4	3.3	0.9	45.6	1.1	1.2	3.9	**0.3**
Aqua- Digby	6.6	13.0	3.8	1.6	67.0	1.6	0.9	5.0	0.6
Aqua- Kilmarnoc Head	**5.0**	6.1	**2.0**	**0.6**	**37.4**	**0.5**	0.8	5.0	**0.3**
Aqua- Lime Kiln	7.5	**6.0**	3.0	0.7	43.0	1.4	0.9	6.3	**0.3**
NS STP	7.0	9.0	2.7	1.6	55.0	**2.8**	0.8	4.6	0.5
NB STP	7.4	9.9	3.7	0.9	49.8	0.7	1.1	**3.4**	0.4
Ferry	13.0	6.0	3.1	0.9	59.0	2.6	**0.6**	**8.7**	**0.3**
Pulp Mill	10.0	**44.6**	**4.6**	**1.9**	62.6	2.4	**1.9**	6.9	**0.8**
Fish Plant	**14.3**	6.3	3.7	0.8	**71.4**	3.7	0.5	7.2	**0.3**

Metal concentrations were generally low: Co, Cd and Ni values were below 2 ug/g, Se and Pb values were below 5 ug/g, and Cu and As values were below 15 ug/g. Values for Mn were also below 15 ug/g, with the exception of the NS low impact area (19 ug/g) and the pulp mill (44.6 ug/g). Values were highest for Zn, with a low of 37.4 ug/g at the Kilmarnoc Head aquaculture site and a high of 71.4 ug/g at the fish plant. Metal concentrations were often lowest at the Kilmarnoc Head aquaculture site and often highest at the pulp mill and fish plant. The less impacted sites had relatively low values in many cases, but they were not always the lowest values among sites.

3.3 Mussel Condition

The results for condition index and mussel leukemia are presented as the mean of 30 replicate mussels from each site (Figure 7). Six individual less impacted sites were sampled: three in NB and three in NS. As there were no significant differences between the values for the individual less impacted sites (one way ANOVA, $F = 0.760$, $p = 0.471$), values were pooled for the six sites.

There was a significant effect of site on condition index (one way ANOVA, $F = 63.536$, $p < 0.0001$). At the zero distance, each treatment site was significantly different than the less impacted site except for the sewage treatment plant in NB.

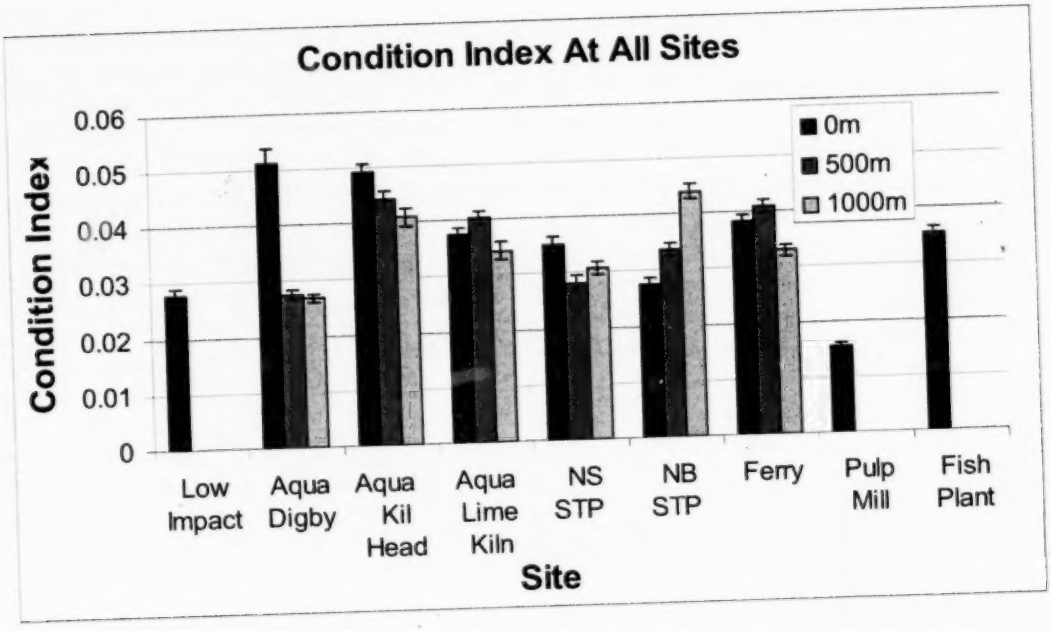


Figure 7: Condition index of mussels (dry weight/shell volume) collected at individual sites. Error bars represent the standard error of the mean

The condition index was generally higher at the aquaculture sites than at the less impacted sites. In terms of Kilmarnoc Head and Lime Kiln, this trend continued up to 1000m away from the facilities. For Digby, there was a significant negative correlation between distance from facility and condition index (linear regression: $R^2 = 0.5061$, $p < 0.001$).

The condition index at the sewage treatment plants, ferry, and fish plant were also higher than the less impacted sites, though values were not as large as those from the aquaculture sites. For the sewage treatment plant in St. Andrews (NB STP), there was a significant positive correlation with distance (linear regression, $R^2 = 0.4947$; $p < 0.001$), the opposite of what was observed for the Digby aquaculture site (NS STP). The pulp mill was the only site with a lower condition index than the less impacted sites.

There was a significant effect of site on the incidence of leukemic individuals (ANOVA, $F = 24.542$, $p < 0.0001$) (Figure 7). Preliminary observation by light microscope revealed that at the less impacted sites, the majority (25 to 29 out of 30 individuals, or 90%) were found to be normal with some individuals having a small number of transitional and leukemic cells.

At the Digby aquaculture site, the leukemic incidence was not significantly different than the less impacted sites (Tukey's post-hoc comparison, $p= 0.914$). At the two remaining aquaculture sites there was a significantly elevated incidence of leukemia (Tukey's post hoc comparison, Kilmarnoc Head: $p = 0.014$;

Lime Kiln: $p < 0.001$). There were a high number of individuals with leukemia at 600m at the Kilmarnoc Head aquaculture site (KH- 600m) and a high number of transitional individuals at the zero distance (KH- 0m). There appeared to be a positive linear relationship between leukemic cells and distance at the Kilmarnoc site. There were a high proportion of individuals with leukemic cells at the zero distance Lime Kiln aquaculture site. There were fewer leukemic cells in more distant samples while the number of transitional cells increased with distance. Less than two thirds of the individuals examined were found to be "normal" at each of these sites.

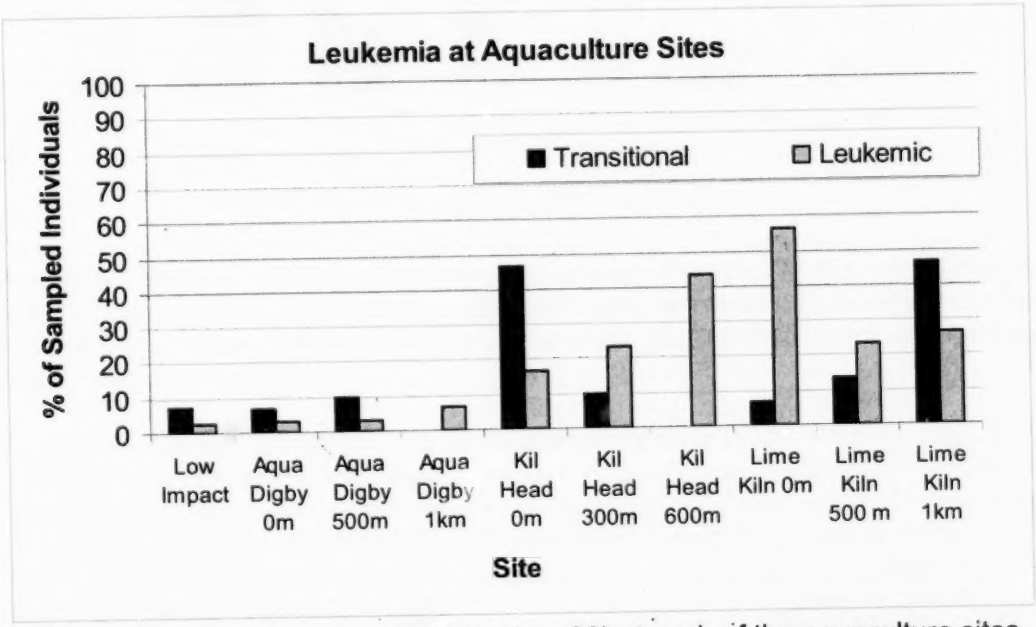


Figure 8: Percent of sample population (n = 30) at each of the aquaculture sites identified as leukemic. Data are grouped according to site (location of aquaculture facility), with three stations (distances) per site.

At the NS sewage treatment plant, the values were similar to less impacted sites (Figure 9). However, at the NB sewage treatment plant over half the individuals at 0m and 1000m were leukemic. At 500 m, there were an elevated number of transitional individuals; therefore the number of normals was still quite small. The NS plant was not significantly different than the less impacted sites while the NB plant had a significant difference from the less impacted sites (Tukey's post-hoc comparison: $p > 0.05$ and $p < 0.0001$, respectively).

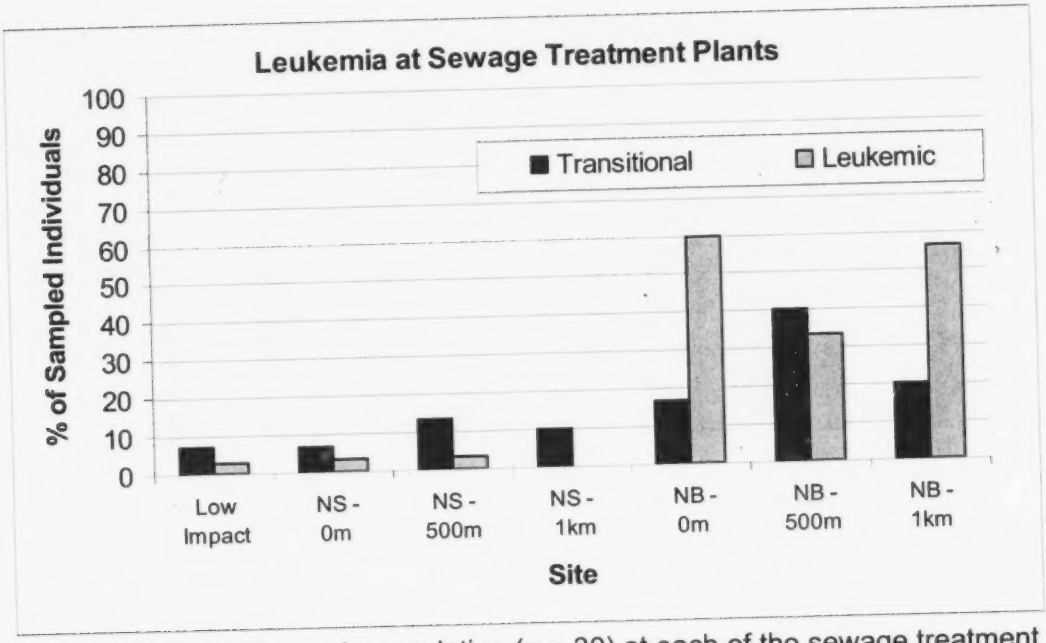


Figure 9: Percent of sample population (n = 30) at each of the sewage treatment plants identified as leukemic. Data are grouped according to site (location of sewage treatment facility), with three stations (distances) per site.

At the ferry station, there was an increasing number of normal individuals with increasing distance from the station. However, even at the 1 km distance, the number of normals was still lower than at less impacted sites. The pulp mill and fish plant had few normals and an elevated number of leukemics and transitionals respectively. Each of these sites was substantially different from the less impacted sites.

The incidence of leukemia, as described above, refers to the number of diseased individuals in the sample population. The intensity of leukemia is the extent of the disease within an individual organism, i.e. the actual proportion of cells that are leukemic.

In eight out of ten samples diagnosed as leukemic by light microscope alone, 50% or more of the cells observed under epifluorescence (after applying the monoclonal antibody technique) were leukemic. Thus, the diagnosis of leukemia by light microscope was confirmed by the monoclonal antibody analysis. The highest intensity of leukemia (meaning the largest number of leukemic cells within an individual) was found at the Lime Kiln aquaculture site, the NB sewage treatment plant and the pulp mill. These are the same sites where the number of individuals with leukemia was highest. Thus, the high intensity of leukemia within individuals correlates well with the high incidence of leukemia in the sample population.

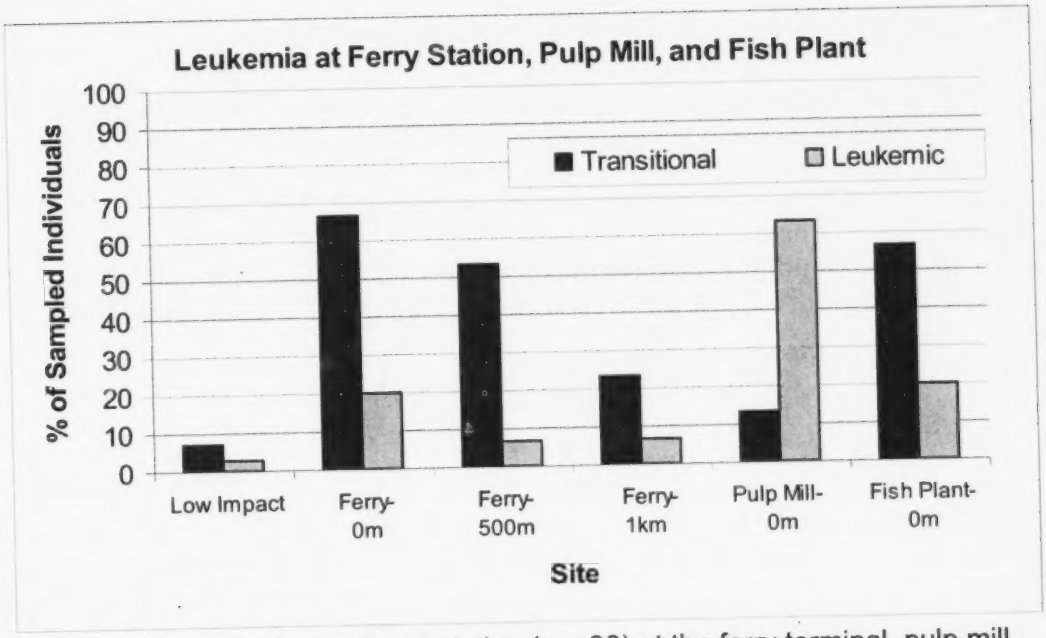


Figure 10: Percent of sample population (n = 30) at the ferry terminal, pulp mill and fish plant identified as leukemic. Data are grouped according to site. For the ferry terminal there are three stations (distances), compared to only one for the pulp mill and fish plant sites.

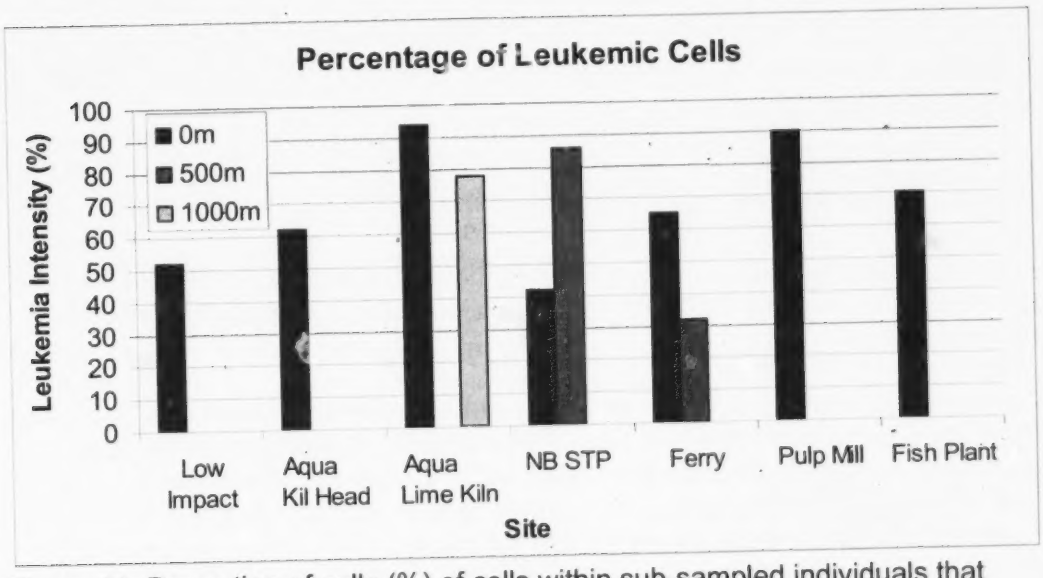


Figure 11: Proportion of cells (%) of cells within sub-sampled individuals that were identified as leukemic.

3.4 Relative Vitellogenin-like proteins in female mussels

Relative vitellogenin-like proteins (vitellins) can be measured indirectly by the assessment of alkali liable phosphates. They indicate vitellogenin levels in the mussels and can be used as biomarkers of estrogenic compounds of endogenous and exogenous origins (Blasie et al. 1999). Mussels from the NB sewage plant site had higher vitellin levels while those from the other sites had lower vitellin levels (Figure 12). The levels at the aquaculture sites were significantly lower (ANOVA: $p < 0.0001$) than those at the less impacted sites.

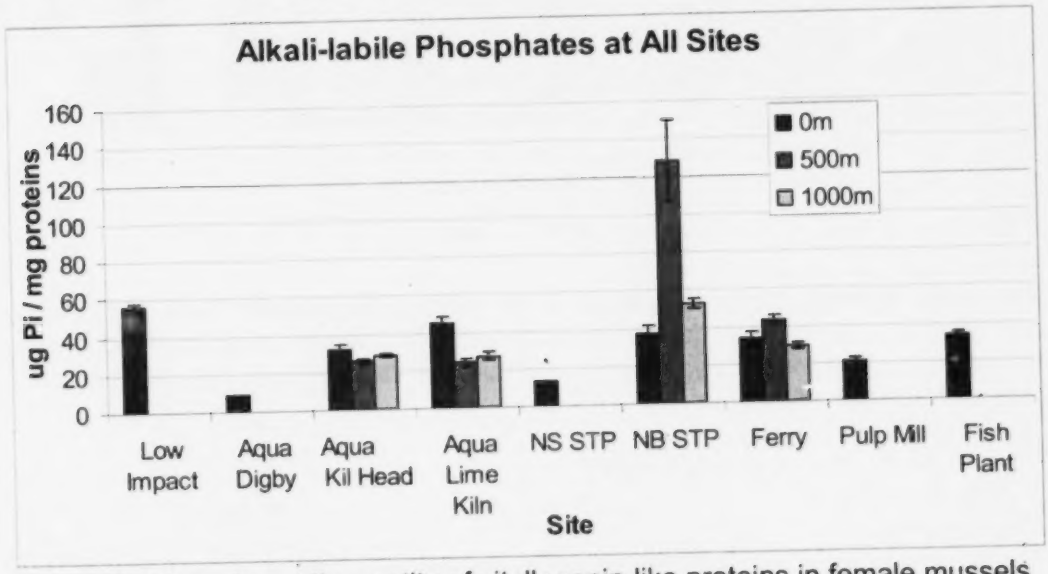


Figure 12: Mean (n = 14) quantity of vitellogenin-like proteins in female mussels at each of the sampling sites.

3.5 Gametogenesis activity (aspartate transcarbamoylase activity)

Aspartate transcarbamoylase is a rate limiting enzyme implicated in the formation of nucleotides from aspartate for the production of DNA for eggs and sperm (Gauthier-Clerc, 2002).

The aspartate transcarbamoylase activity at the Digby aquaculture site (NS) was lower than at the less impacted site, while the aspartate transcarbamoylase activity at the NB aquaculture sites was higher than at the less impacted site. The overall effect of site on gametogenesis activity was significant (ANOVA $p < 0.0001$). The NB sewage treatment plant activated this enzyme activity. The effect was variable at the other sites.

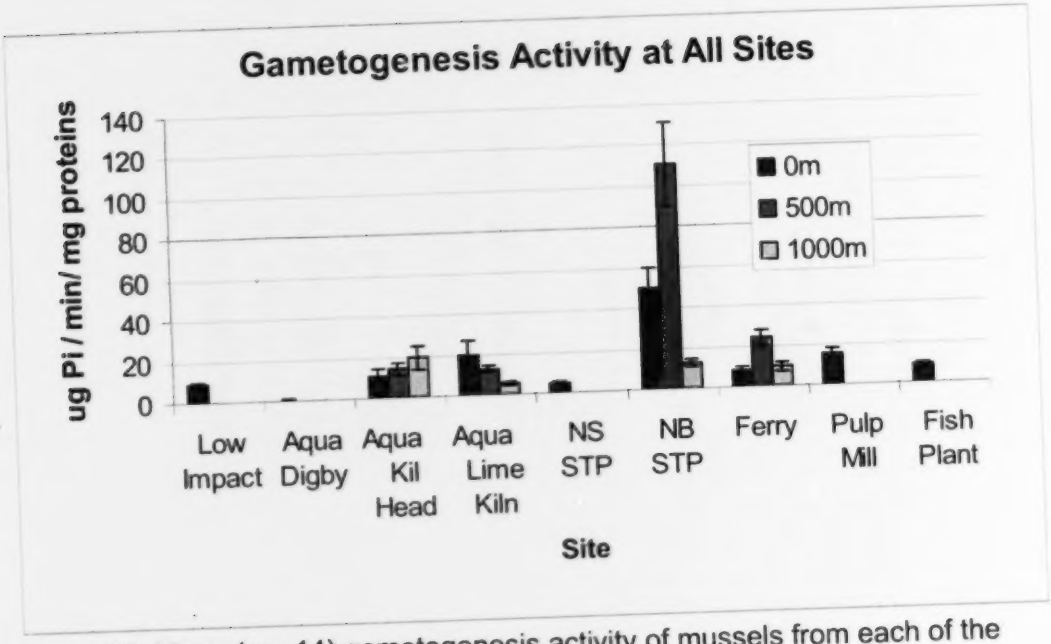


Figure 13: Mean (n = 14) gametogenesis activity of mussels from each of the sampling sites.

3.6 Coprostanol levels

Coprostanol levels were significantly elevated at the NS sewage treatment, ferry, pulp mill, and aquacultures sites relative to the less impacted sites (ANOVA: $p < 0.0001$) (Figure 14).

Coprostanol is produced by the reductive metabolism of cholesterol by the gut flora in mammals. Coprostanol abounds in feces and is, thus, considered a biomarker of exposure to domestic sewage (Gagné et al. 2002). The less impacted station contains more Coprostanol than the NB sewage treatment plant and less than the NS sewage treatment plant.

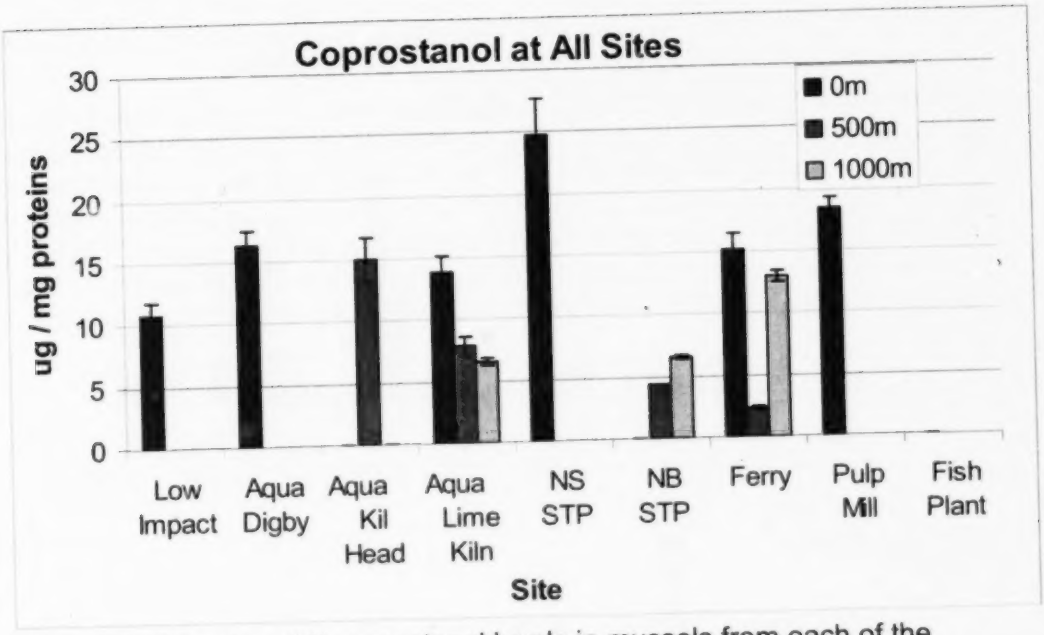


Figure 14: Mean (n = 14) coprostanol levels in mussels from each of the sampling sites.

3.7 Cholesterol levels

The presence of cholesterol, the precursor of sexual steroids, has been shown to indicate increased feeding, increased steroidogensis, the action of endocrine disrupting compounds, or the blockage of sexual steroid synthesis (Gagné et al. 2002).

The cholesterol level at the NS aquaculture site was not significantly different from the less impacted sites. The NB aquaculture sites displayed an increased cholesterol level relative to the less impacted sites. In terms of Kilmarnoc Head, there was an increase in cholesterol with distance and at the Lime Kiln site, there was a decrease in cholesterol with distance. The same was seen for gametogenesis. The level at the NS sewage treatment plant was similar to less impacted sites, while the NB plant level was elevated. Levels were also elevated at the ferry terminal and pulp mill.

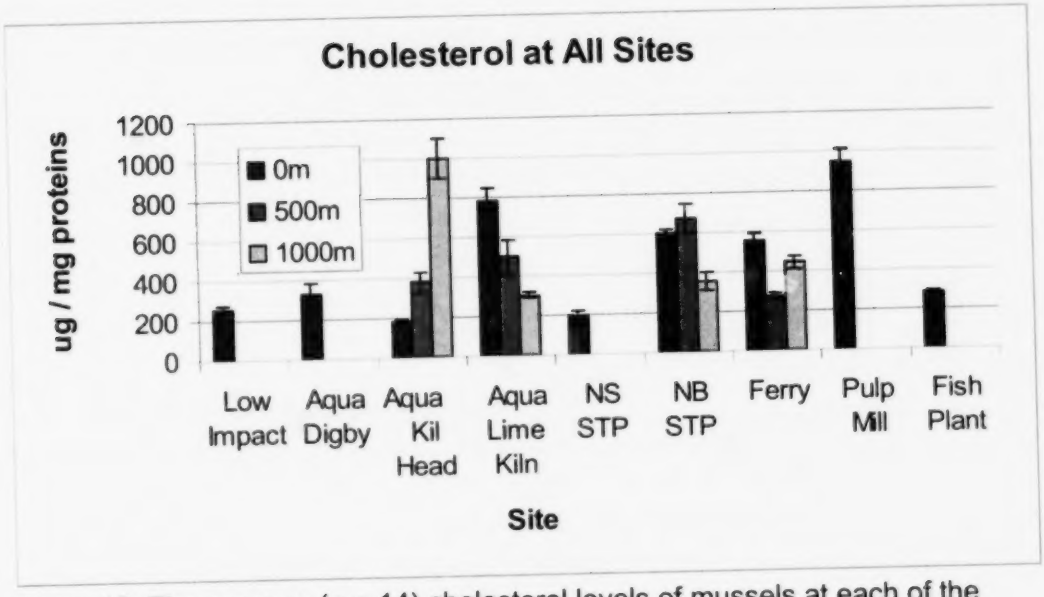


Figure 15: The average (n = 14) cholesterol levels of mussels at each of the sampling sites.

3.8 Yeast Estrogen Screen (YES assay)

The sediment concentrations used were 14.8 – 20 mg/ml, 34.4 – 40 mg/ml, 86 – 100mg/ml, and 172 – 200 mg/ml. Where there is an increase in estradiol activity with increasing sediment concentration, it may be stated that the estrogenicity is due, at least in part to, the presence of the sediment itself. Sediment samples were extracted with water, this represents bioavailable estrogenicity. Figure 16 presents the results from the water-extracted sediment.

The screening of NB 500m and NS 1000m there was substantial increases in estrogenic activity with increasing sediment concentrations.

Estrogenicity increased with sediment concentration (from 0.3% at 17 mg/ml to 26% at 180 mg/ml) at the NS less impacted site NS 500m. At the less impacted site NS 0m, the observed increase was slight (up to 5.7% at 180 mg/ml). At the less impacted site NS 500m, there was actually a slight decrease in estrogenicity with increasing sediment concentration (from 3.3% at 17 mg/ml to 2.0 at 180 mg/ml). This is the opposite of what would be expected if the observed estrogenicity was attributable to the sediment itself.

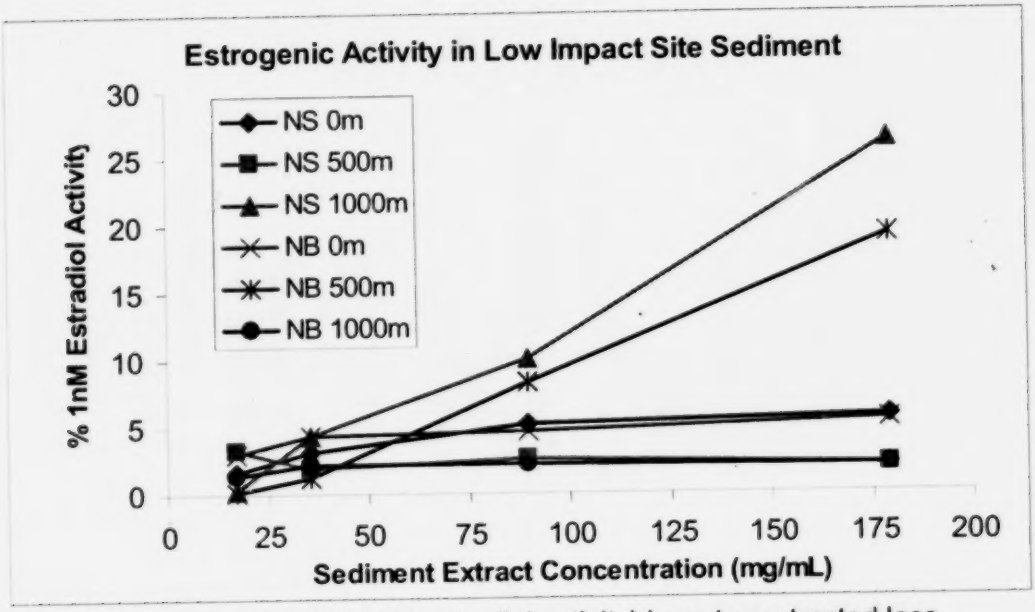


Figure 16: Estrogenicity (% 1nM estradiol activity) in water-extracted less impacted site sediments as measured by theYES assay.

In NB, the estrogenicity detected in water-extracted samples from the less impacted site (NB 1000m) was approximately 2% at the various sediment concentrations tested. An increase in estrogenicity was seen with increasing sediment concentration for the less impacted site (NB 500m), up to 19.2% at 180 mg/ml. A smaller increase, up to 5.5%, was seen for the less impacted site NB 0m.

Where the measured estrogenicity does not increase with increasing sediment concentration (NS 0m, 500m and NB 0m, 1000m) the observed estrogenic activity cannot be attributed to the sediment. Thus, the remaining treatment sites from this study were compared to NS 500m and NB 500m, the sites where there was an increase in estrogenicity with increasing sediment concentration.

At the NS aquaculture site, there was an increase in estrogenic activity 0m (D0) from that of the aquaculture site (Figure 17). Very little estrogenic activity was seen in the water-extracted samples from the 500 m and 1000m sites. The highest amount of estrogenicity was seen at 0m (D-0) (3% at 18 mg/ml and 11.6% at 185 mg/ml).

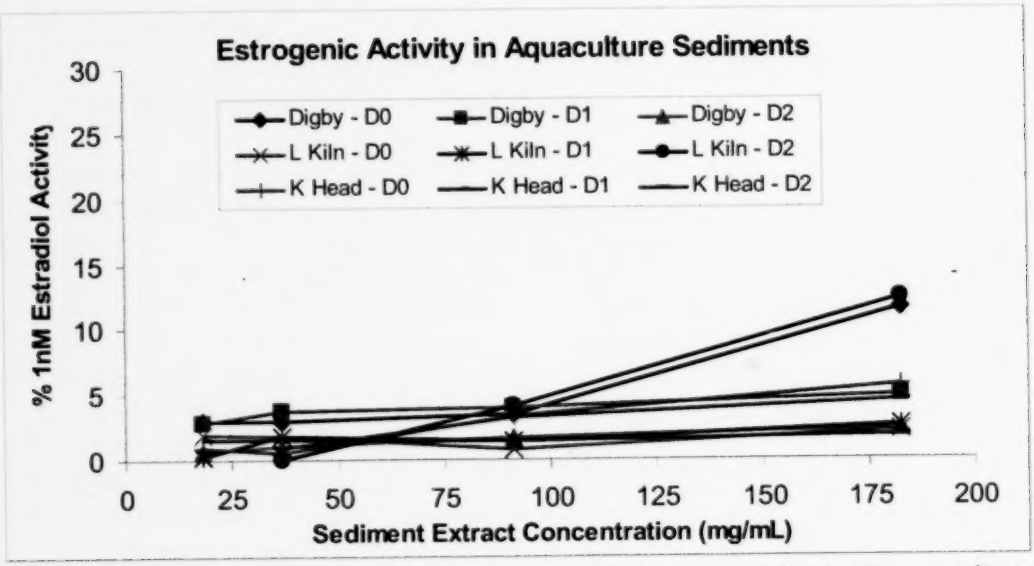


Figure 17: Estrogenicity (% 1nM estradiol activity) in water-extracted aquaculture site sediments as measured by the YES assay.

At the NB aquaculture site, estrogenicity was found to be 200 mg/ml at all three distances in the water extracted samples. At 0m and 500 m (D-0 and D-1), there was approximately 2% estrogenicity at 185 mg/ml. For the furthest distance, there was approximately 12% estrogenicity at 185 mg/ml. In all cases, the amount of estrogenicity is half, or less, than that observed at NS less impacted and NB less impacted sites.

A small amount of estrogenicity was seen at 0m from the NS sewage treatment plant (up to 12.2% at 195 mg/ml) (Figure 18). This is comparable to the highest values for the aquaculture sites and is approximately half the values observed at NS Low 2 and NB Low 2. All sewage treatment sites displayed little estrogenic activity as measured by the YES assay.

There was very little estrogenicity at any of the ferry, fish plant, or pulp mill sites (Figure 19). At the ferry site values were consistently below 2% and there was no increase with increasing sediment concentration. Fish plant values were below 2% and again, there was no increase. The pulp mill showed an increase in estrogenicity with increasing sediment concentration, but only up to 6% at 185 mg/ml.

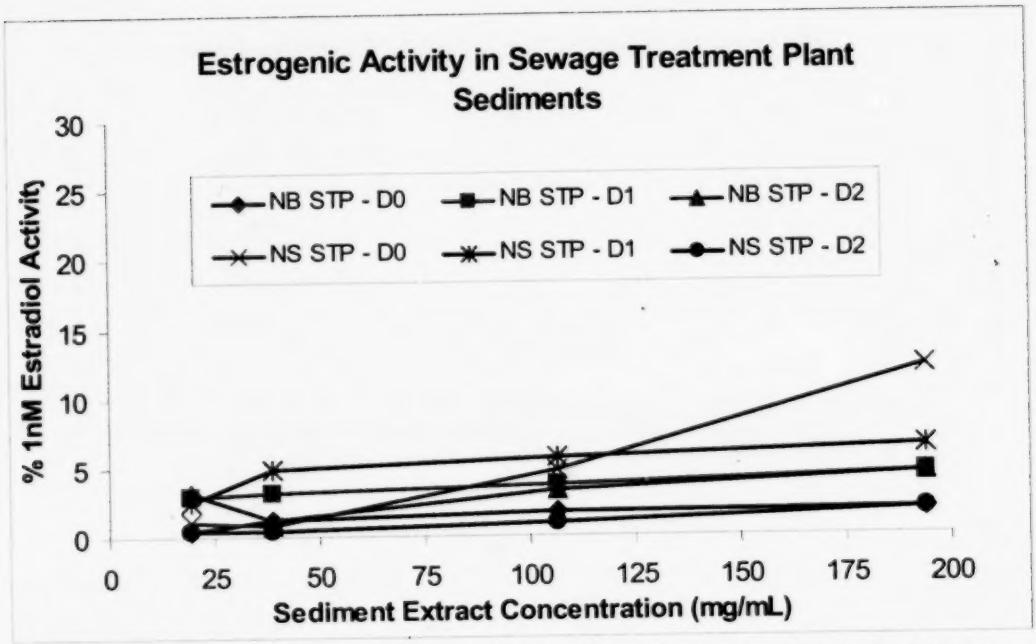


Figure 18: Estrogenicity (% 1nM estradiol activity) in water-extracted sewage treatment plant sediments as measured by the YES assay.

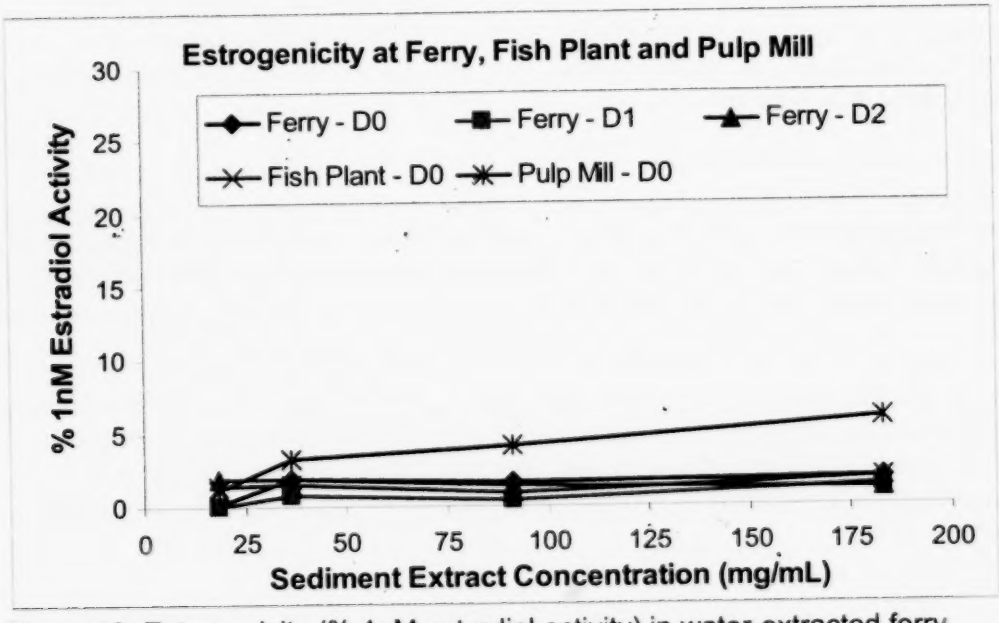


Figure 19: Estrogenicity (% 1nM estradiol activity) in water-extracted ferry terminal, fish plant and pulp mill sediments as measured by the YES assay.

4.0 DISCUSSION

4.1 Organic Contaminants in Mussel Tissues

The concentrations of PCBs at the ferry terminal, Lime Kiln aquaculture, sewage treatment and fish plant sites were substantially higher than those found by the Gulfwatch in 1998, where PCB levels ranged from not detected to 1.005 ng/g in mussels from less impacted sites in NB and from undetectable to 0.53 ng/g in NS (Jones et al. 2001). The values of chlorinated pesticides as a group determined by the Gulfwatch and converted to wet weight ranged from 0.87 ng/g to 1.05 ng/g for NB and 0.77 ng/g to 0.9 ng/g for NS (Jones et al. 2001). These values are much lower than the values in the current study. The values from the Gulfwatch were originally reported on a dry weight basis, and were converted here to wet weight for comparison (assuming an 85% moisture content). The difference in values may partly be attributed to the wet/dry difference and subsequent conversion, or the fact that the Gulfwatch locations included what could be considered as 'background' sites.

The tissue residue guideline for DDT for the protection of wildlife consumers of aquatic biota (ng/g diet ww), is 14 ng/g (CCME, 2001). This value was exceeded at five of the six treatment sites in NB including: the Lime Kiln aquaculture site (18 ng/g), the sewage treatment plant (22 ng/g), ferry terminal (43 ng/g), pulp mill (29 ng/g) and fish plant (61 ng/g).

The tissue concentration of a particular contaminant within an organism at the threshold of sublethal effects may be termed the critical body residue (CBR) (Jones et al. 1998). In a study using aquatic macro-invertebrates and fish, the CBR for PCB's was found to be 45.3 $\pm$ 6.54 ng/g (Dillon, 1984). The PCB values in the current study at the fish plant and ferry terminal are higher than this CBR, indicating that the mussels at these two sites were likely impacted by the presence of PCB's.

4.2 Metal Contaminants in Mussel Tissues

The metal concentrations detected in mussel tissues by this study were very similar to those by the Gulfwatch in 1998 (Jones et al. 2001). Although zinc concentrations were the highest of the metals reported, they were about one-third the reported concentration that has been reported to cause a slight reduction in gametogenesis (245 ug/g) (Myint and Tyler, 1982).

The slightly elevated concentration of lead in ferry terminal mussels may possibly be related to the historical use of lead as a component of fuel.

Cadmium values were 0.5 – 1.9 ug/g and were similar across treatments. All concentrations measured in this study were several orders of magnitude less

than the critical body residue (CBR) for cadmium (324 ug/g dry weight) determined by Dillon in 1984. Our results were similar to those of the Gulfwatch which reported cadmium concentrations in mussels of 0.88 to 1.5 ug/g in NB and 1.60 to 2.73 ug/g in NS (Jones et al. 2001). Body residues of 75 ug/g Cd have been shown to have no impact on gametogenesis (Myint and Tyler, 1982), and residues of 150 ug/g dry weight had no observable effect on clearance, ingestion, food absorption, respiration or growth (Poulsen et al. 1982). The large difference between the previous values reported in the literature and the currently observed tissue concentrations indicates that cadmium levels are probably not affecting the mussels at the various sites.

Copper concentrations were very similar across treatments (3-7 ug/g). They were slightly elevated at the ferry terminal, up to almost 12 ug/g. This could be related to the use of antifoulant, of which Cu is a component, on ships. In the Gulfwatch mussels, concentrations ranged from 5.15 ug/g to 13 ug/g in NB and from 5.33 ug/g to 6.73 ug/g in NS (Jones et al. 2001). Numerous studies have demonstrated that laboratory exposure of mussels to varying degrees of copper contamination has an overall effect on their health. Concentrations in the growth medium as low as 20 ug/L cause mussels to significantly reduce the amount of time they filter food from water. Shell growth can be inhibited at concentrations as low as 2 – 10 ug/L (Manley, 1983; Manley et al. 1984). In a field study, it was demonstrated that lipid peroxidation products (evidence of oxidative stress and damage) were correlated with increasing exposure to heavy metals (Moore,1985; Viarengo et al. 1990, 1991). The CBR by Dillon (1984) for copper was 217.5 ug/g dry weight, a full two orders of magnitude higher than any measured in this study. In a more conservative study, a mussel-specific CBR was set at 28 ug/g dry weight because whole body concentrations as low as 28 – 42 ug/g elicited significant reductions in gametogenesis and vitellogenesis (Myint and Tyler, 1992). This lower CBR value by Myint and Tyler is closer to the Cu concentrations seen across treatments in this study.

Although the contaminant data from the Gulfwatch is extensive, only a small number of studies have attempted to delineate the threshold tissue concentration at which contaminant levels have an adverse effect (Jones et al. 1998). When examining the extent of organic contamination in the current study, it can be seen that tissue concentrations of certain organics (DDT, its derivatives and dieldrin) were ubiquitous and the former were elevated above tissue residue guidelines at 5 of 6 treatment sites in NB. Other chemicals that were present at detectable levels only at a few sites, were still present at the less impacted site in NB, where there were no identified sources of anthropogenic chemical inputs, other than sparse residential buildings. Where metal CBR's are available (data are few), those reported in the scientific literature were often higher than the tissue concentrations reported in the current study. However, CBR's for the organics, especially PCB's, indicated that tissue concentrations in the current study may be of concern, especially at the fish plant and at the ferry terminal in NB.

4.3 Mussel Condition

The relation of wet weight to shell volume is traditionally used by shellfish biologists to assess bivalve condition (Widdows, 1985). In this case, dry weight was used to reduce the potential variability of wet weights if mussels were drained to a different degree prior to weighing.

One might expect to find higher condition index at the less impacted locations, however, that was not the case in this study. With the exception of the pulp mill site, at all of the treatment sites mussels had condition indices which were elevated above the less impacted sites. The condition index values at each 0m sites was plotted against the mussel tissue contaminant concentration at the corresponding site for all metal and organic chemicals tested. Linear regression analysis revealed significant relationship between the effect variable (condition index) and the contaminant concentrations for dieldrin ($p < 0.05$; $R^2 = 0.604$) and manganese ($p < 0.05$; $R^2 = 0.505$). It is possible that other factors in addition to those tested (such as antibiotics, therapeutants, nutrients, food pellet particles and fish feces) were present at the aquaculture sites, and acted as growth enhancers rather than growth inhibitors. It is also probable that rather than being a sole function of contaminant levels, the differences in condition index are due to environmental variables such as temperature, nutrient status and plankton biomass. These are among the primary factors known to impact growth and condition index (Dickie et al. 1984; Mallet and Carver, 1993).

The exact cause of leukemia in mussels is still unknown. It has been suggested that the disease is virally induced as bivalves from unpolluted sites have it, though at low frequency (Oprandy et al. 1981, Oprandy and Chang, 1983). Other proposed causative factors include mussel nutrition and immune function.

Although the cause of leukemia in molluscs has yet to be determined, some hypothesize that there is a relationship between leukemia occurrence and the presence of environmental contaminants. Mussels may filter contaminants which are carcinogenic and/or tumour promoting in nature (Barker et al. 1997). A strong correlation between high PCB levels and leukemia in the soft shell clam (*Mya arenaria*) was found in New Bedford Harbour, Massachusetts. Cancer was found in 22-70% of the clam population tested (Reinisch et al. 1984; Harper et al. 1994), and PCB levels in mussels (separate studies) were as high as 30mg/g dry weight (Bergen et al. 1993, Nelson et al. 1995). These high tissue concentrations were correlated with the high sediment and water PCB concentrations.

It has been documented that soft shell clams (*Mya arenaria*) from the Massachusetts area have background levels (that is, leukemia occurrence in pristine areas) of 10.8 to 27.5% (Reinisch, et al. 1984). The expected background occurrence of leukemia in mussels is 5-10% (Dr. Carol Reinish, pers. comm., 2003). Hence, the observed values at the less impacted sites in both NS and NB in the current study were well within background levels. At the

Digby aquaculture site and the Digby sewage treatment plant (both in NS), the values were similar to the less impacted sites, and thus also fell within background levels. However at the sewage and aquaculture sites in NB, the leukemia incidence was elevated. It was also elevated at the rest of the NB treatment sites including the ferry station, pulp mill and fish plant.

Although PCB levels were not elevated at the aquaculture sites, they were elevated at the ferry (70 ng/g), pulp mill (15 ng/g) and fish plant (40 ng/g), where leukemia rates were significantly elevated above background levels. In addition to PCB's, these sites had elevated concentrations of chlordane, and DDT and its derivatives.

In an attempt to determine if leukemia could be induced in normal mussels by exposure *in situ* to environmentally contaminated sites, normal wild blue mussels from Pictou, NS were exposed to less impacted sites and to industrialized areas including a steel plant and buried city dump, a secondary sewage treatment plant, a coal generated electrical power plant, and a pulp mill (St. Jean et al. in press). The caged transplant experiment took place during the ice-free season of 2002. Normal mussels were collected from a pristine less impacted site, caged, and transplanted into contaminated sites. It was found that mussels exposed to anthropogenic effluents had a significantly greater chance of developing leukemia than those animals caged at the less impacted sites (St. Jean et al. submitted). This study contributes to the growing evidence that heavily contaminated environments correlate with leukemia in bivalves (Reinisch et al. 1984; Miosky et al. 1989; Harper et al. 1994).

4.4 Biomarkers of Reproduction and YES Assay

The production of vitellins, the major egg-yolk protein, has been shown to be controlled by estradiol (Li et al. 1998). Moreover, vitellin production was induced in the haemolymph of the softshell clam (*Mya arenaria*) on exposure to estrogens in the laboratory and in the field near municipal effluent (Blaise et al. 1999). This corresponds to the vitellins (as indicated by the measurement of alkali-labile phosphates) in the current study being elevated at the NB sewage treatment plant. The fact that this did not happen until 500m is not entirely surprising. It has been previously shown that decreased vitellin expression was observed close to Montreal's urban effluent site but it became elevated at further distances (Gagné et al. 2001).

The lower vitellin levels at the remaining sites may indicate an androgenic or anti-estrogenic response. The lower vitellin levels at the aquaculture sites relative to the less impacted sites are interesting because another aquaculture study found elevated vitellogenin levels in Atlantic salmon in response to treatment with 0.25 mg/kg ivermectin, a sea-lice control chemical (Ucan-Moran et al. 2002).

The Saguenay Fjord in the St. Lawrence River of Quebec has many sources of anthropogenic pollution including aluminium, pulp and paper production, municipal waste, and pesticide residues from farming practices. Known contaminants include polyaromatic hydrocarbons (PAHs), dioxins, mercury and cadmium (Pelletier and Canuel, 1988). It was found that *Mya arenaria* from this region displayed much higher levels of alkali-labile phosphates than the less impacted site (Blaise et al. 1999).

It is well known that municipal effluent may contain elevated levels of estrogenic substances. Gagné (et al. 2001) exposed freshwater mussels (*Elliptio complanata*) to municipal effluent in the St. Lawrence River in Quebec, 5 km downstream from an effluent plume. It was found that Vg levels increased significantly in both the hemolymph and the gonad after exposure to the effluent. From this observation, researchers concluded that municipal effluents contain bioavailable xenoestrogens at levels sufficient to elicit effects in freshwater mussels. A study on the soft-shell clam (*Mya arenaria*) from the Saguenay Fjord also found that animals collected from sites influenced by municipal wastewaters had higher levels of vitellin (Blaise, et al. 2002). Furthermore, vitellin levels were positively correlated with DNA damage and EROD activity (7-ethoxyresorufin O-deethylase) and negatively correlated with metallothionein-like protein levels. It is known that the reproduction cycle causes energy-demanding physiological changes in animals.Those authors suggest changes may have rendered the defence mechanisms less successful in averting a toxic effect, as evidenced by in the increase in DNA damage. Thus the relationship between increased vitellin levels and these variables suggests that reproduction can influence the susceptibility of clams towards some contaminants (Blaise, et al. 2002, 2003).

Overall, the correlations between the reproductive biomarkers and the measured contaminant levels were quite variable. In addition to the variability in levels of reproductive biomarkers at various sites, the concentrations were generally low. For example, concentrations of vitellin were below 60 ug Pi/mg protein in all samples except for the NB sewage treatment plant (which was 130 ug Pi/mg protein). At the peak reproductive period (usually in June) concentrations in mussels from an unaffected site may average 280 ug Pi/mg protein (Blaise et al. 1999). It is possible that had the samples been taken just prior to the reproductive peak, then patterns in the observed perturbations may have been easier to detect.

It is known that chemical inputs into the marine environment at the various treatment sites may potentially accumulate in the marine sediment. If any of the accumulated chemicals possessed estrogenic activity, then the YES assay should have detected it in the sediment samples tested. The results of the YES assay in the current study indicate that the estrogenicity observed at the treatment sites cannot be differentiated from the less impacted sites. Little is known about the exposure of organisms in the aquatic environment to sediment-associated (xeno-biotic) estrogenic compounds. However, at the levels observed

in the current study, estrogenicity in the sediment itself does not seem to be a major concern (Dr. Kent Burnison, pers. comm., 2003).

5.0 Summary and Conclusions

Several effect variables were measured throughout the course of this study including condition index, leukemia, and reproductive biomarkers (vitellogenin-like proteins, gametogenesis, coprostanol and cholesterol). Leukemia prevalence was not elevated at sewage treatment and aquaculture sites in NS, yet it was significantly elevated at each of the activity sites in NB, compared to the less impacted sites. The highest numbers of individuals with leukemia were found in NB at the aquaculture sites, the sewage treatment plant, and the pulp mill. The latter treatment sites were correlated with elevated concentrations of organic contaminants. Condition indices were highest at the aquaculture sites and were also significantly elevated compared with the less impacted sites, the sewage treatment plants, ferry, and fish plant. The reproductive biomarkers showed great variability. Vitellin levels were significantly elevated at the NB sewage treatment plant and significantly decreased at the remaining treatment sites. Gametogenesis was also significantly elevated at the NB sewage treatment plant, and variable at the remaining treatment sites. Coprostanol and cholesterol also had variable responses. Both measures were elevated in many cases (including the NB sewage treatment plant), but not at all of the distances sampled for each of the treatment sites. Overall, there were significant differences between the less impacted sites and the treatment sites for all the reproductive biomarkers measured.

When examining the extent of metal and organic contamination reported in this study, it can be seen that in several cases the tissue concentrations were below available Critical Body Residues (the threshold concentration at which adverse effects may occur) reported in the primary literature. In the case of Cu, values were two orders of magnitude lower than those previously reported. Thus, it is not surprising that not all response variables examined (ie: condition index, leukemia and reproductive biomarkers) correlated significantly with the metal and organic contaminants analysed.

There is the potential that other contaminants not analysed or other chemical parameters (i.e; pH, temperature, dissolved oxygen), alone or in combination, may be responsible for the elevated adverse effects identified in these samples. Further biomarker investigations may be important in determining the environmental quality in specific locations throughout the Bay of Fundy. Further research,, including the analysis of other parameters, is necessary in order to better understand the potential cause(s) of the adverse effects identified at the aquaculture sites, sewage treatment plant, fish processing plant, and pulp mill in NB.

The cumulative evidence indicates that there are physiological effects on mussels which can be attributed to substances released from industrial activities, most notably ferry terminals and fish plants, but also sewage treatment plants and aquaculture sites. There did not seem to be strong evidence of endocrine effects, however reproductive impairment may be occurring. Although there were elevated concentrations of contaminants, there was no correlation between contaminant concentration and any biological responses.

Literature Cited

Barker, C.M., R.J. Calvert, C.W. Walker, and C. Reinisch. 1997. Detection of Mutant p53 in Clam Leukemia Cells. *Experimental Cell Research.* 232:240-245.

Blaise, C., F. Gagné, J. Pellerin and P.D. Hansen. 1999. Measurement of vitellogenin-like properties in the hemolymph of *Mya arenaria* (Saguenay Fjord, Canada) : a potential biomarker for endocrine disruption. *Environmental Toxicology* 14(5):455-465.

Blaise C, Gagné F, Salazar M, Salazar S, Trottier S, Hansen P-D. 2003. Experimentally-induced feminisation of freshwater mussels after long-term exposure to a municipal effluent. *Fresenius Environmental Bulletin.* 12(8):865-870.

Blaise, C., F. Gagné, J. Pellerin, P.D. Hansen and S. Trottier. 2002 Molluscan shellfish biomarker study of the Saguenay Fjord (Quebec, Canada) with the soft-shell clam, *Mya arenaria. Environmental Toxicology.*17:170-186.

Bergen, B.J., W.G. Nelson, J.W. Pruell. 1993. Bioaccumulation of PCB congeners by blue mussels (*Mytilus edulis*) deployed in New Bedford Harbor, MA. *Environmental Toxicology and Chemistry.* 12:1621-1681.

Burnison K.B., A. Hartmann, A. Lister, M.R. Servos, T. Ternes and G. Van der Kraak. 2003. A toxicity identification evaluation approach to study estrogenic substances in hog manure and agricultural runoff. *Environmental Toxicology and Chemistry.* 22(10):115-122.

Canadian Council of Ministers of the Environment (CCME). 2001. Canadian Environmental Quality Guidelines. Includes: Canadian Water Quality Guidelines for the Protection of Aquatic Life and Canadian Sediment Quality Guidelines for the Protection of Aquatic Life.

Chase, M.E., S.H. Jones, P. Hennigar, J. Sowles, G.C.H. Harding, K. Freeman, P.G. Wells, C. Krahforst, K. Coombs, R. Crawford, J. Penderson and D. Taylor. 2001. Gulfwatch: monitoring spatial and temporal patterns of trace metal and organic contaminants in the Gulf of Maine (1991-1997) with the blue mussel, *Mytilus edulis* L. *Marine Pollution Bulletin.* 42(6):491-505.

Cooper K.R., R.S. Brown, P.W. Chang. 1982. The course and mortality of a hemopoetic neoplasm in the soft shell clam *Mya arenaria. Journal of Invertebrate Pathology.* 39:149-157.

DeGuise, S., S.D. Shaw, J.S. Barclay, J. Brock, A. Brouwer, E. Dewailly, P.A.

Fair, M. Fournier, P. Grandjean, L.J. Guillette Jr., M.E. Hahn, C. Koopman-Esseboom, R.J. Letcher, A. Matz, R.J. Nostrom, C.R. Perkins, L. Schwacke, J.U. Skaare, J. Sowles, D.J. St. Aubin, J. Stegeman, and J.E. Whaley. 2001. Consensus Statement: Atlantic Coast Contaminants Workshop 2000. *Environmental Health Perspectives.* 109(12):1301-1302.

Dickie, L.M., P.R. Boudreau and K. R. Freeman. 1984. Influences of stock and site on growth and mortality in the blue mussel *(Mytilus edulis). Canadian Journal of Fisheries and Aquatic Sciences.* 41:134-140.

Dillon, T.M. 1984. Biological consequences of bioaccumulation in aquatic animals: An assessment of the current literature. U.S. Army Corps of Engineers, Long-Term Effects of Dredging Operations Program. Technical Report D-84-2. June 1984. 69p.

Eckelbarger KJ, Davis CV. 1996. Ultrastructure of the gonad and gametogenesis in the eastern oyster, *Crassostrea virginica.* I. Ovary and oogenesis. *Marine Biology.* 127:79-87.

Gagné, F., J. Pellerin, C. Blaise, and S. Gauthier-Clerc. 2002. Variation in the biochemical properties of female gonads and vitellins in the clam *Mya arenaria* at contamianted sites in the Saguenay Fjord. *Marine Environmental Research.* 53:295-310.

Gagné F, Blaise C, Lachacne B, Sunahara GI, Sabik H. 2001. Evidence of coprostanol estrogenicity to the freshwater mussel *Elliptio complanata. Environmental Pollution.* 115:97-106.

Gagné, F., C. Blaise, M. Salazar, S. Salazar, and P.D. Hansen. 2001. Evaluation of estrogenic effects of municipal effluents to the freshwater mussel *Elliptio complanata. Comp. Biochem. Physiol.* 128C:213-225.

Gauthier-Clerc, S., J. Pellerin, Ç. Blaise, F. Gagné. 2002. Delayed gametogenesis of *Mya arenaria* in Saguenay fjord (Canada) : a consequence of endocrine disruptors ? Comp Biochem Physiol Part A()

Harper, D.M., D.A. Flessas, C.L. Reinisch. 1994. Specific reactivity of leukemia cells to polyclonal anti-PCB antibodies. *Journal of Invertebrate Pathology.* 64:234-237.

Herries, D.G. 1967. The simultaneous estimation of orthophosphate and carbamoylphosphate and application to the aspartate transcarbamoylase reaction. *Biochemica and Biophysica acta.*136:95-98.

Jones, S.H., M.Chase, J. Sowles, P. Hennigar. N. Landry, P.G. Wells, G.C.H.

Harding, C. Krahforst and G.L. Brun. 2001. Monitoring for toxic contaminants in *Mytilus edulis* from New Hampshire and the Gulf of Maine. *Journal of Shellfish Research*. 20(3):1203-1214.

Jones, S.H., M. Chase, J. Sowles, P. Hennigar, W. Robinson, G. Harding, R. Crawford, D. Taylor, K. Freeman, J. Pederson, L. Mucklow, K. Coombs. September 1998. The First Five Years of Gulfwatch, 1991-1995: A Review of the Program and Results. Gulf of Main Council on the Marine Environment.

Kavloc, R.J., G.P. Daston, C. DeRosa, P. Fenner-Crisp, L. Earl Gray, S. Kaattari, G. Lucifer, M. Luster, M. J. Mac, C. Maczka, R. Miller, J. Moore, R. Rolland, G. Scott, D. M. Sheehan, T. Sinks and H.A. Tilson. 1996. Research needs for the risk assessment of health and environmental effects of endocrine disruptors: A report of the U.S. EPA-sponsored workshop. *Environmental Health Perspectives*. 104(supplement 4):715 – 740.

Leavitt, D.F., J.M. Capuzzo, D. Miosky, R. Smolowitz, B. Lancaster, C.L. Reinisch. 1990. Incidence of hematopoietic neoplasia in *Mya arenaria*: monthly monitoring of prevalence and indices of physiological condition. *Marine Biology*. 105: 313-321.

Li, Q., M. Osada, T. Suzuki, and K. Mori. 1998. Changes in vitellin during oogenesis and effect of estradiol on vitellogenesis in the Pacific oyster *Crassostrea gigas*. *Invertebrate Reproduction and Development*. 33:87-93.

Mallet, A.L. and C.E. Carver. 1993. Temporal production patterns in various size groups of the blue mussel. *Journal of Experimental Marine Biology and Ecology*. 170:75-89.

Manley, A.R. 1983. The effects of copper on the behaviour, respiration, filtration, and ventilation activity of *Mytilus edulis*. *Journal of the Marine Biology Association of the U.K.* 63:205-222.

Manley, A.R., L.D. Gruffydd and P.C. Almada-Villela, 1984. The effect of copper and zinc on the shell growth of *Mytilis edulis* measured by a laser diffraction technique. *Journal of the Marine Biology Association of the U.K.* 64:417-427.

Mathieu M. 1987. Utilization of aspartate transcarbamylase activity in the study of neuroendocrinal control of gametogenesis in *Mytilus edulis*. *Journal of Experimental Biology*. 241:247-252.

Miosky, D.L., R.M. Smolowitz, C.L. Reinisch. 1989. Leukemia cell-specific

protein of the bivalve mollusc *Mya arenaria*. *Journal of Invertebrate Pathology*. 53:32-40.

Moore, M.N. 1985. Cellular responses to pollutants. *Marine Pollution Bulletin*. 16:134-139.

Muise, B.C. and Associates. June 2000. Chemical Use by the Eastern Canadian Aquaculture Industry. Environment Canada Pollution Prevention Division Environmental Management and Technology Section. Atlantic Region.

Myint, U.M. and P.A. Tyler. 1982. Effects of temperature, nutritive and metal stressors on the reproductive biology of *Mytilus edulis*. *Marine Biology*. 67:209-223.

Nelson, W.G., B.J. Bergen, D.J. Cobb. 1995. Comparison of PCB and trace metal bioaccumulation in the blue mussel , *Mytilus edulis* and the ribbed mussel, *Modiolus demissus*, in New Bedford Harbor, MA. *Environmental Toxicology and Chemistry*. 14:513-521.

Oprandy, J.J., P.W. Chang. 1983. Bromodeoxyuridine induction of hematopoetic neoplasia and retrovirus activation in the soft shell clam, *Mya arenaria*. Journal of Invertebrate Pathology. 42:196-206

Oprandy, J.J., P.W. Chang, A.D. Pronovost, K.R. Cooper, R.S. Brown, And V.J. Yates. 1981. Isolation of a viral agent causing hematopoetic neoplasia in the soft shell clam, *Mya arenaria*. Journal of Invertebrate Pathology. 38:45-51.

Pelletier, E. and G. Canuel. 1988. Trace metals in surface sediment of the Saguenay Fjord, Canada. *Marine Pollution Bulletin*. 19:336-338.

Poulsen, R.T., H.U. Riisgard and F. Mohlenberg. 1982. Accumulation of cadmium and bioenergetics in the mussel *Mytilus edulis*. *Marine Biology*. 68:25 -29.

Purdom, C.E., P.A. Hardiman, V.J. Bye, N.C. Eno, C.R. Tyler and J.P. Sumpter. 1994. Estrogenic effects of effluent from sewage treatment works. *Chemical Ecology*. 8:275-285.

Reinisch, C.L., A.M. Charles, A.M. Stone. 1984. Epizootic neoplasia in soft shell clams collected from New Bedford Harbor, MA. *Hazardous Waste*. 1:73-81.

Servos, M., G.J. Van der Kraak and M. Wade. 2001. Scientific assessment of endocrine disrupting substances in the Canadian environment. *Water Quality Research Journal of Canada*. 36(2):171-173.

St. Jean, S., E. Pelleter and S.C. Courtenay. 2002. Hemocyte functions and bacterial clearance affected *in vivo* by TBT and DBT in the blue *mussel Mytilus edulis. Marine Ecology Progress Series.* 236:163-178.

St. Jean, S., S.C. Courtenay, and C.L. Reinisch. Leukemia introduction in the blue mussel in Pictou, NS. Submitted to *Marine Biology*.

Ucan-Moran, F., W. Ernst, R. O'Dor, J. Sherry and S.C. Johnson. 2002. Endocrine disrupter effect of ivermectin in atlantic salmon (*Salmo salar* L.) Poster Presented at the 35th Annual Symposium of the Society of Toxicology of Canada. Montreal, December 2002.

U.S. EPA Risk Assessment Forum. February 1997. Special Report on Environmental Endocrine Disruption: An Effects Assessment and Analysis. Technical Report EPA/630/R-96/012.

Viarengo, A., L Canesi, M. Pertica, G. Poli, M.N. Moore and M. Orunesu, 1990. Heavy metal effects on lipid peroxidation in the tissues of *Mytilus galloprovincialis* Lam. *Comp. Biochem. Physiol.* 97C:37-42.

Viarengo, A., L Canesi, M. Pertica, D.R. Livingstone and M. Orunesu. 1991. Age-related peroxidation in the digestive gland of mussels: The role of the antioxidant defense system. *Experientia.* 47:454-457.

Viarengo, A., L Canesi. 1991. Mussels as biological indicators of pollution. *Aquaculture.* 94:225-243.

White, P., C. Blaise, J. Rasmussen. 1997. Detection of genotoxic substances in bivalve molluscs from the Saguenay Fjord (Canada), using the SOS Chromotest. *Mutation Research.* 392:277-300.

Widdows, J. 1985. Physiological measurements. In: Bayne, B.L., D.A. Brown, K. Burns, D.R. Dixon, A. Ivanovici, D.R. Livingstone, D.M. Lowe, M.N. Moore, A.R.D. Stebbing and J. Widdows, eds. The effects of stress and pollution on marine animals. New York: Praeger Pulishers. Pp. 3-39.

Appendix A

Table A: Location of the field sites in NS.

				Decimal	Decimal
Location	Distance	Lat/Long	Degrees	Minutes	Lat/Long
Less Impacted Site	1000m	N	44	41.844	44.6974
		W	-65	-38.92	-65.64863333
	500m	N	44	42.149	44.70248333
		W	-65	-38.64	-65.64403333
	0m	N	44	42.248	44.70413333
		W	-65	-38.48	-65.64136667
Sewage Treatment Plant	1000m	N	44	37.25	44.62083333
		W	-65	-45.21	-65.75356667
	500m	N	44	36.783	44.61305
		W	-65	-45.11	-65.75176667
	0m	N	44	36.62	44.61033333
		W	-65	-45.45	-65.75741667
Aquaculture Site	1000m	N	44	38.659	44.64431667
		W	-65	-45.35	-65.75585
	500m	N	44	38.914	44.64856667
		W	-65	-45.43	-65.75716667
	0m	N	44	39.162	44.6527
		W	-65	-45.46	-65.75773333

Appendix B

Table B: Location of the field sites in NB.

Location	Distance	Lat/Long	Degrees	Decimal Minutes	Decimal Lat/Long
Indian Point (STP)	1000m	N	45	4.165	45.06942
		W	-67	-2.894	-67.0482
	500m	N	45	4.008	45.0668
		W	-67	-2.412	-67.0402
	0m	N	45	4.321	45.07202
		W	-67	-2.106	-67.0351
Kilmarnoc Head (Aquaculture)	600m	N	45	7.57	45.12617
		W	-67	-1.895	-67.0316
	300m	N	45	7.747	45.12912
		W	-67	-1.858	-67.031
	0m	N	45	7.987	45.13312
		W	-67	-1.966	-67.0328
Lime Kiln (Aquaculture)	1000m	N	45	4.154	45.06923
		W	-66	-48.604	-66.8101
	500m	N	45	4.136	45.06893
		W	-66	-48.666	-66.8111
	0m	N	45	3.806	45.06343
		W	-66	-49.482	-66.8247
Grand Manan Ferry	1000m		45	3.39	45.0565
			-66	-47.995	-66.7999
	500m	N	45	2.794	45.04657
		W	-66	-48.327	-66.8055
Less Impacted (Dead Man's)	3	N	45	2.984	45.04973
		W	-66	-46.173	-66.7696
	2	N	45	3.108	45.0518
		W	-66	-46.118	-66.7686
Pulp Mill	0m	N	45	7.207	45.12012
		W	-66	-45.978	-66.7663